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Biotechnology Conference:
Diagnostics '87

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A detailed review is given of selected topics presented at this conference held in December 1987 at Cambridge, UK. Topics include thin-layer technology, single-step immunoassays, rapid microbial assays, diagnostic applications of DNA probes, DNA probe *in situ* hybridization assays, new amperometric biosensors, and application of electrochemical methods to immunoassays.

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BIOTECHNOLOGY CONFERENCE: DIAGNOSTICS '87

INTRODUCTION

The conference entitled "Diagnostics '87" was the third in the Cambridge Series on Biotechnology organized by IBC Technical Services, London, UK. This focused and informative conference, held on 10 and 11 December, 1987, at Cambridge, was planned by C.R. Lowe, Biotechnology Center, University of Cambridge, UK, and C.P. Price, Addenbrooke's Hospital, University of Cambridge School of Clinical Medicine, UK.

There were 177 attendees at this conference, with the majority from the UK. However, 11 European countries were also represented as well as the US, Australia, and India. Seventy-five percent of the participants were from industrial organizations while 25 percent were from academia.

The conference program consisted of the following topics:

- Diagnostics – the impact of the new technologies
- Clinical diagnostics
- Reagent-strip technology
- Thin-film technology
- Single step immunoassay systems
- Enzyme-amplified immunoassays
- Fluorescent and luminescent immunoassay
- Rapid microbial assay technology
- Diagnostic applications of gene probe technology
- DNA probe technology – *in situ* hybridization assays
- New amperometric biosensors
- Application of electrochemical methods to immunoassays
- Magnetic resonance techniques in diagnostic medicine.

The last decade has seen dramatic improvements in the technologies underlying the development of diagnostic procedures. This progress is linked with the discovery of, and improvements in, conceptually new approaches such as amplified enzyme-linked immunoassay, DNA probe technology, and biosensors. The aim of the conference was to review the current status and likely developments in these new technologies, allow comparisons between competitive technologies and assess their likely impact on the market for diagnostic products.

Keywords Great Britain, (GC/RW)

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These new developments in diagnostic technologies are likely to have a profound effect on the way analytical procedures are performed, with a move away from specialized central facilities towards the site of analysis in real-time. This trend will not be restricted to clinical diagnostics but may also apply to agricultural, horticultural, veterinary, pharmaceutical, and biotechnology-based analytical procedures. This conference was designed to address these implications and thus had appeal to research and technical personnel, product development, and marketing staff.

A fairly detailed review of selected topics will be presented in this report.

THIN-LAYER TECHNOLOGY: MONOLAYERS TO MULTI THIN FILMS

This topic was discussed by J.F. Padday (Research Division, Kodak Limited, Wealdstone, Harrow, UK). Padday said that in the photographic process small grains of silver halide become developable when exposed to visible light. The development process reduces only the exposed siver halide to silver and in the vicinity of that grain the molecules of developer become oxidized. In color photography the oxidized developer is coupled with a further organic molecule, the color coupler, to form a dye. A true, colored image is achieved by confining three different photoinduced processes to each of three separate thin layers of gelatin, wherein each is sensitive to different colors and each produces a complementary color (cyan, yellow, or magenta). There are many ways of using the elements of this process according to whether the image required is negative, positive-reflection print, transparency, or instant print.

A main feature of all these options is that the reduction of silver and the consequent production of color must be confined to the layer and must not diffuse sideways significantly. An example of this thin-layer process is demonstrated in Figure 1, which shows the disposition of the 15 layers that make up the pack of an instant color photo-material. In this product each of the photosensitive layers – blue, green, and red – forms magenta, yellow, and cyan dyes, which are water soluble and diffuse through several other layers before they become anchored permanently on the mordant layer. The same thin-film technology has now been adapted to produce, in a much simpler way, a method for measuring the amount of certain constituents of human body fluids, which are present in blood plasma, serum and urine.

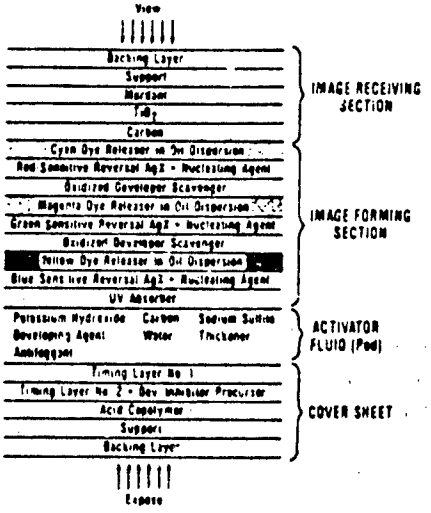


Figure 1. Instant color film layers.

Constituents of Human Blood

It is well known that the major constituents of human blood plasma range over four orders of magnitude of both quantity and molecular size. Also, the proteins are numerous and vary widely in concentration. In the EKTACHEM process developed at Kodak for assaying the smaller inorganic ions such as potassium, sodium, chloride, bicarbonate, etc., concentrations are measured by electrochemical means using reversible electrodes in conjunction with the Nernst equation. In principle, the target ion is made the potential controlling factor by interposing within the cell an organic polymer membrane that is permeable to the target ion only. Thus, the reversible membrane potential becomes dependent solely on the activity of the target ion.

Padday said that the activity coefficient linking activity and concentration depends on the total concentration of all ionic species according to the Debye Huckel theory of interaction of ions in an electrolyte solution. At normal levels of ions an activity coefficient significantly less than unity has to be applied to the Nernst equation, according to Padday. Also the fluid to be analyzed by the methods described below must be freed from particulate material such as blood cells, usually by centrifugation.

The Thin Layer Concept

Padday said that thin layers as described in the photographic system are usually coated onto a flexible support (paper or film base), and consist of the appropriate substances embedded in a gelatin matrix originally coated from aqueous solution. The dried-down thickness is less than 50 micrometers and can be as thin as 10 nanometers. Water is taken up avidly by the dried-down layer which then swells. The total amount of water required to achieve this swelling is very small and of the order of microliters per square centimeter.

Thin-layer technology has been applied to clinical analysis by using very similar enabling chemistry but with far fewer layers than in the photographic system, according to Padday. The analytical unit is the slide, which is about the size and shape of a mounted color transparency. Each slide is used once only and requires an autoanalyzer machine to effect the measurement.

An analytical slide is comprised of a plastic mount which sandwiches a piece of the coating. The coated layers are supported on a transparent film base, thus allowing the color of the analytical reactions to be measured from the back so that it may be free from any masking colors present in the sample solution.

The top layer of a clinical slide is invariably a spreading layer which often includes functional factors within it. Below are more functional layers. Bewteen these layers and the base support are coated the electrodes that measure potential. These are omitted on colorimetric slides. The disposition of layers is shown in Figure 2 together with the liquid metering tip. The volume of liquid to be dispensed is normally 10 microliters, and accurate metering is achieved (independent of variable surface tension) by the attraction of the spreading layer pulling away the suspended volume, as shown in the figure.

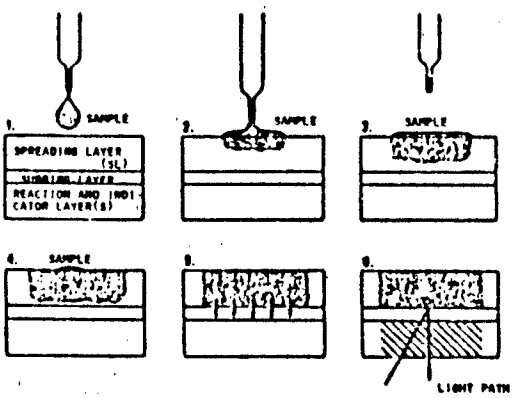


Figure 2. EKTACHEM sample metering.

As in photography the coated layers are not formed exclusively with gelatin. There are four types of layers defined according to overall function. These types, shown in Figure 3, are: (1) filtration, (2) selective absorption, (3) anchoring reactants, and (4) kinetic optimization.

Selective absorption is taken to include selective transport of a target ion through a membrane. With reference to the spreading layer, we note that the layer is formed with particles of titania or baryta embedded in a matrix of cellulose acetate some 0.3 mm thick. A part of thin-film technology developed includes the interweaving of hydrophobic polymer layers with the other more hydrophyllic layers such as those of gelatin.

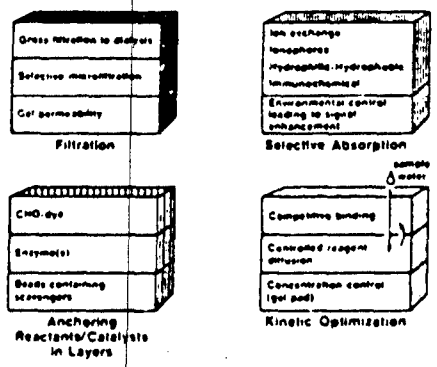


Figure 3. Thin layer functions.

Colorimetric Measurement with a Slide

The end product of reactions within the color-producing slides is a dye, the amount of which is proportional to the amount of target substance initiating the reaction chain. Color is measured from the back by reflection spectroscopy as shown in Figure 4. The reflection density as defined in the Figure 4 equations 2b and 1b do not obey the Lambert Beer law, which applies to transmission densities only, but follows a more complex relationship originally developed by Kubelka and Munck. The incident light enters from the rear and is reflected off the white inner surface of the spreading layer, thereby greatly enhancing the signal. The transformation of signal to concentration of target substance is achieved by determining the parameters of equation 3c (Figure 4) separately and embedding them with calibration constants into appropriate software to produce a final print out of the required analysis.

$$R = \frac{I_R}{I_0} \qquad \text{(Equation 1b)}$$

$$D_R = \log_{10} \frac{1}{R} \qquad \text{(Equation 2b)}$$

$$C = A_0 + A_1 \cdot D_R \qquad \text{(Equation 3b)}$$

However,

$$C = A_0 + A_1 \cdot g_1(D_R) + A_2 \cdot [g_2(D_R)]^2 \qquad \text{(Equation 3c)}$$

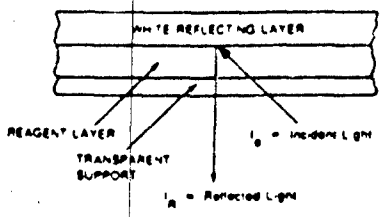


Figure 4. Thin layer reflectance.

The detailed chemistry of a selection of such analytical slides is given below.

Potentiometric Measurements (Potassium Ions). The mechanism of this method is best explained by reference to the measurement of potassium ion concentration using the EKTACHEM slide which is shown diagrammatically in Figure 5. The slide is comprised of two half-cells, each with a silver-silver chloride reversible electrode. Above each is coated an internal reference layer of saturated KCl and, finally, on top is a hydrophobic polymer layer permeable to some water and to potassium ions which are carried by the ionophore, valinomycin. As seen in the figure the two half-cells are connected by a paper bridge which is sandwiched between plastic sheeting. Small drops of the patient's serum and of a reference liquid containing a known concentration of potassium are placed each on one half-cell as shown. Connections between the two silver electrodes provide a very high impedence potential which, again, is transformed electronically to evaluate the concentration difference between it and the reference liquid.

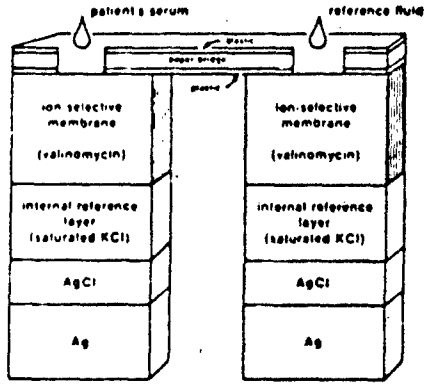


Figure 5. A thin layer cell, EKTACHEMSLIDE.

As is well known, the potential measured by the silver-silver halide electrode is the Galvani potential. The electrode is not easily poisoned except by the other less soluble halide ions, bromide and iodide. The layer of potassium chloride provides a constant potential between the electrode and, also, suitable conductivity between electrode and semipermeable membrane. The semipermeable membrane is a very thin layer of polymer with the potassium ionophore dissovoled in it. A type of Donnan membrane potential is set up which in about a minute reaches a stable constant potential as a measure of potassium activity. The ionophore valinomycin, "solubilizes" potassium ions in the organic polymer by encasing each ion with an organic shell.

Valinomycin is a ring compound composed of six alternating peptide and ester linkages. The diameter of the center nearly equals the unhydrated diameter of the potassium yet appears to have little affinity for the sodium ion. Thus the potassium is solubilized and all other ions

excluded (or nearly so). This solubility difference is termed "selectivity," and is a thermodynamic quantity. The membrane potential is again measured as the Galvani potential difference between each side of the membrane. To avoid excessive build-up of potential inside the membrane and to encourage uptake of the potassium by the valinomycin, an organic ion of negative charge is added to the membrane.

The slide is strictly a device for measuring the potential of an ion-selective membrane. The potassium level of many samples of human serum and of human urine have been compared, according to Padday, with the results obtained by the flame photometry method, and good correlation was obtained with the EKTACHEM data.

Chloride, Carbonate, and Sodium Ions

The potentiometric slide for chloride ions is very similar to the potassium slide but with the difference that the polymer membrane containing valinomycin is replaced with a semipermeable membrane of cellulose acetate which allows the passage of chlorine ions but excludes the larger interfering ions of bromide and uric acid.

In the case of sodium ions, many types of sodium-measuring electrodes have been reported, according to Padday. Most of these are based on the glass electrode in preference to a polymer ionophoric membrane of the type used for potassium. The sodium slide of the EKTACHEM system is based on the half-cell as for chloride ion measurement. The top layer of this half-cell is a thin layer of polymer containing the ionophore methyl monensin. According to Padday the selectivity of this ionophore is sufficient to exclude potassium ions from the membrane so that the potential measured is derived from the difference in activity of sodium ions only, each side of the membrane.

Measurement of Carbon Dioxide as Carbonate Ions

The carbon dioxide uses selective absorption via ion-exchange to measure the divalent carbonate ion. The carbon dioxide half-cell requires an extra layer in the thin-layer package. This extra layer, which uses a tetra-alkyl ammonium sulphate salt and buffered with salicylate, is an ion-exchange membrane which immobilizes all the larger organic ions. The carbonate ion is not immobilized and passes into the ion-selective membrane beneath. The same quarternary salt ion-exchanger in the presence of trifluoroacetophenone dissolved in a vinyl resin now becomes an ionophore for the doubly charged carbonate ion. As with the potassium electrode system the underside of the selective membrane is connected to a silver/silver chloride with a intermediate reference layer but of saturated sodium chloride instead of potassium chloride. As with the potassium slide, these other electrodes measure the potential difference between two half-cells connected together by a paper bridge as described in Figure 5.

The Colorimetric Assay Method

The main problem associated with producing a color by the thin-film method, according to Padday, is to ensure that other materials present in the very small sample do not interfere with the target material and its chain of reaction. Padday then described three typical examples of the many colorimetric slides:

1. Triglycerides. Bioreactions are initiated by immobilized enzymes and end up with the production of hydrogen peroxide which, with peroxidase, oxidizes the almost colorless leuco form of the dye to a deep turquoise color. The first step is that of breaking down the lipoproteins into free glyceride esters and this is brought about by a surfactant present in the top layer which itself is a spreading layer. Also present in this layer is a lipase, prepared from the yeast *Candida rugosa*, which is free of the protease chymotrypsin. This lipase attacks all three bonds of the glyceride este, to leave glycerol and free fatty acids. The glycerol then diffuses through a layer of ascorbate oxidase which converts the ascorbate ion into a non-interfering molecule and leaves the glycerol untouched to enter the bottom layer. In this layer, the glycerol is successively converted to dihydroxyacetone phosphate and hydrogen peroxide. Hydrogen peroxide then produces dye to give a measure of glycerol present as triglycerides.

2. Calcium. The calcium assay depends for its efficacy on the metachromatic changes that take place when a dye ion changes its immediate environment. In this slide the substituted dye, arsenazo III, is sufficiently soluble in water media to move around, and in so doing becomes immobilized on the mordant layer above it. Within this layer the arsenic acid group of the azodye is able to form a complex which greatly disturbs the pi-electron system of the chromophore, as is well known in the photographic process. The effect of the calcium is thus not to produce dye but to change the color of the dye that is already there. The reflectance density is measured at the wavelength of the perturbed dye which, through the appropriate calibration transform, provides a measure of the calcium ion concentration.

3. Creatinine and Ammonia. Unlike the previous two assays, the creatinine assay depends on the formation of ammonia by the reaction shown in Figure 6. As some ammonia is likely to be present this must be determined at the same time by performing a parallel assay but with the creatinine iminohydrolase enzyme absent from the ammonia comparison slide. The top layer is a spreading layer under which lies the creatinine iminohydrolase. The ammonia released in this layer passes through a polymer semipermeable membrane which excludes other basic components from the dye layer at the bottom of the pack. The dye used here is a triphenyl methane indicator dye, bromophemol blue, which like other similar dyes becomes intensely colored only in alkaline solution. Thus, the amount of ammonia is measured from the density of blue in the reflectance spectrum. The amount of

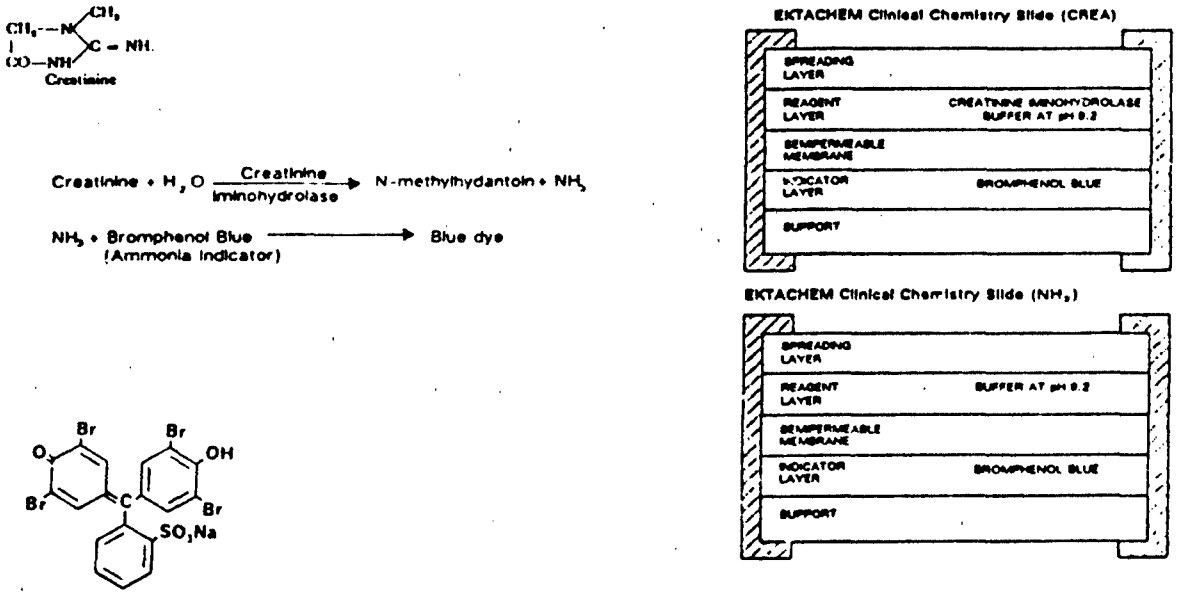


Figure 6. Creatinine ammonia.

creatinine is obtained from the difference between the ammonia and the creatinine slides.

Rate Methodologies

The rate at which the above reactions take place is readily followed with time without any apparatus changes, according to Padday. Thus the thin layer technology has been adapted to measure enzyme concentration by timing the rate of change of color. The principle is demonstrated in Figure 7, which shows the EKTACHEM amylase slide. As with the other technologies very small amounts of the patient's sample are required – i.e., 11 microliters.

The amylase catalyzes the hydrolysis of the dyed starch which itself is the spreading layer to create mobile dyed saccharides. These diffuse into a gelatin mordant layer which binds the dyed atomic fragments. The dye reflectance density is measured through the back, using the starch layer as a nearly white reflector (the majority of starch-dye being hidden from view by the starch) to provide a measure of amylase efficiency and hence concentration.

Thus, Padday reviewed the thin-film EKTACHEM methods by showing examples of the technologies and their underlying principles. The range of tests available by each of the methods described above is shown in Table 1. Clearly, the principles allow for many other test slides as methods with corresponding pathways become available. In many of the tests the principles discovered in the development of photography have been applied to the EKTACHEM system

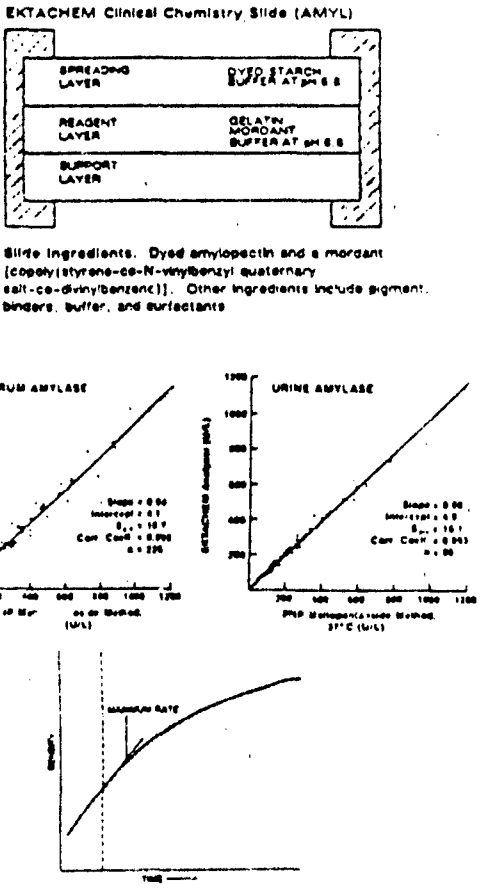


Figure 7. EKTACHEM amylase slide.

Table 1. Analysis methodologies.

Colorimetric	Potentiometric	Rate
Albumin	Carbon dioxide	Alanine aminotransferase
Ammonia	Chloride	Alkaline phosphatase
Billrubin	Potassium	Amylase
Blood urea nitrogen	Sodium	Aspartate aminotransferase
Calcium		Creatline kinase
Cholesterol		Creatine kinase MB
Creatinine		Creatinine
Glucose		gamma Glutylamyltransferase
Hdl cholesterol		Lactate dehydrogenase
Magnesium		Lipase
Phosphorous		Theophylline
total Protein		
Triglycerides		
Uric acid		

SINGLE-STEP IMMUNOASSAY SYSTEMS

The availability of new, simple to use, immunoassay technology is an important factor responsible for the emergence of the Alternate Site diagnostics market. A report on a novel Immunostrip system was presented by T.S. Baker (Department of Research & Development, Boots-Celltech Diagnostics Limited, Slough, UK). In his introduction, Baker said that in recent years the diagnostics industry has witnessed the emergence of a new and important market sector known as the Alternate Site market. This market encompasses non-laboratory-based testing in the physician's office, side-ward or outpatient department, home-use self-testing and nonhuman testing such as applications in horticulture, agriculture and veterinary practise. Market forces responsible for this trend are complex but, importantly, it is the advances in assay technology which are having a major impact on the expansion of the Alternate Site market, according to Baker.

The non-laboratory application of a diagnostic product requires that the test be simple to perform, involves less hands-on time, and be more robust than a corresponding laboratory-based test. A series of new product formats aimed at the Alternate Site market have attempted to meet these requirements. Many of the new test formats designed for this market are antibody-based, owing to the versatility of immunoassay systems for measuring a wide range of analytes, especially those involving monoclonal antibodies in "user-friendly" delivery systems.

According to Baker, some of the more dynamic expansions in the immunodiagnostics Alternate Site sector have been in qualitative, noninstrument-dependent immunoassays such as for home-use pregnancy and fertility testing. In this respect, four basic types of heterogeneous immunoassay systems have found widespread application; these are:

- Particle agglutination immunoassays
- Immunoassays using antibody-coated tubes or paddles
- Immunoassays using filter device
- Immunochromatography devices.

Heterogeneous immunoassays are usually multireagent, multistep procedures which often require considerable and precise manipulation in the laboratory to achieve adequate performance, according to Baker. There are examples of products for each of the above formats where the manufacturer has sought to simplify assay design to improve ease of use and achieve applicability in the Alternate Site market. However, according to Baker, with the possible exception of certain immunochromatography device-based tests, no kit is yet available in which a heterogeneous immunoassay has been reduced to a single-step procedure. He said that usually, several manipulative operations with reaction timings are required and, unless manufacturers' instructions are followed precisely, mistakes can easily be made. Therefore, there is much room for improvement of test format. Baker said that in designing a test for the Alternate Site market the following specifications should be considered.

- One-step operation
- Rapid and self-timed test – 5 to 15 minutes
- Wide application
  - competitive immunoassays
  - noncompetitive immunoassays
  - two-site immunoassays
  - haptens or large molecular weight antigens
- Flexibility of results
  - qualitative – read by eye results
  - quantitative – when interrogated with simple instrumentation
  - stable color end-point
- Stable at ambient temperature (greater than 18 months' shelf life)
- Easily disposable – self-contained, nontoxic reagents
- Suitable format for mass production.

Baker said that a novel diagnostic test system is under development in his laboratory which he believes meets most, if not all, of the above requirements. The system is based on an immunochromatographic test strip or 'Immunostrip' device. In the following paragraphs, a report of the principles behind this new assay system and its potential application are presented.

Principle of the Immunostrip System

Features of the immunochromatographic test strip: According to Baker, the immunostrip has been conceived as an integral device which contains all the necessary reagents for carrying out automatically any one of a variety of formats of heterogeneous enzyme immunoassay. The essential elements of the immunostrip are (1) a small bilbulous paper strip ($15.5 \times 1 \times 0.1$ cm) on which immunochemicals have been deposited in a stable, dry chemistry form and (2) at one end of the paper strip, a reservoir containing some 2 ml of a developing solution, which is physically separated from the dry chemistry component by a rupturable membrane.

The immunochemicals are laid down as discrete transverse zones along the length of the strip. Near the reservoir end is a sample location zone for receiving an aliquot of the sample to be tested. In the mid-region of the strip is a zone called the measuring location at which the test result is observed. Near the end of the strip distal to the reservoir is an indicator zone which acts as a procedural control and end-of-test signal.

The immunostrip is operated by applying an aliquot of the sample to the sample location, and the test is initiated by application of finger pressure to the reservoir. This action ruptures the reservoir membrane and allows developing solution to contact the paper strip. Under capillary action, developing solution is drawn along the paper strip from one end to the other. As it does so, sample and resolubilized immunochemicals mix and migrate along the strip, reacting as they do so in a predetermined manner. Some 10 minutes later, a blue color appears at the indicator zone, signalling that the test can be read at the measuring location. The presence or absence of a blue color or the intensity of the blue color, at the measuring location indicates the test result.

Baker said that seven key features of the Immunostrip system are as follows:

1. The spatial order of the different immunochemical reagents in zones along the strip dictates the reaction sequence of the incorporated immunoassay.

2. The distance between different zones on the strip controls reaction times of each stage of the assay.

3. At least one of the reagent zones on the strip is an immobilized immunochemical. This allows the automatic separation of the bound and free fractions of the complementary binding partner to the immobilized immunochemical, since ongoing development of fluid along the strip will wash nonbound species out of the immobilized zone.

4. The dry chemistries on the paper strip include an enzyme-labeled species, while the developing solution contains a substrate for this enzyme. The chemical nature of the substrate, together with properties of the strip or dry reagents on the strip, prevents the premature interaction of substrate with enzyme during the initial stages of strip development.

5. The substrate has been chosen to yield a blue-colored product which does not migrate along the strip and is relatively stable.

6. The paper strip matrix has been carefully selected to give a minimal nonspecific binding of the various immunochemicals and sample constituents.

7. The method of immobilizing immunochemicals into certain zones on the strip results in a high surface area of active immobilized species relative to the small reaction volumes in the adjacent interstices of the paper matrix. This allows for very rapid reaction kinetics.

According to Baker, these key features of the immunostrip create the potential for high-performance, robust immunoassays to be carried out and allow for versatility in assay design within the constraints of the test device. In order to further describe the immunostrip system, Baker gave an example of an actual test for human Chorionic Gonadotropin (HCG).

Baker's Description of an HCG Immunostrip.

Figure 8 is a simplified diagram of an immunostrip for detecting the presence of HCG in a urine sample; such a system could form the basis for a simple pregnancy test. The configuration shown is a two-site immunoassay format, which would be applicable to other tests requiring this format. The diagram indicates the relative positions of the reservoir containing developing solution, the sample location, the measuring location and the indicator zone. In addition to these zones is a reagent zone just downstream of the sample location which contains soluble horse radish peroxidase (HRP)-labeled anti-HCG antibody in a dry, stable form. A second anti-HCG antibody, complementary to the labeled antibody is present in the measuring location in immobilized and dry, stable form. This antibody had been previously passively adsorbed to a microparticulate carrier such that, when deposited at the measuring location, the microparticles become enmeshed between the fibers of the paper and are unable to migrate when the strip is developed. The indicator zone contains immobilized enzyme to act as a procedural control and end-of-test indicator. Finally, the developing solution contains hydrogen peroxide (H_2O_2) substrate and 3,3',5,5' tetramethylbenzidine (TMB) cofactor in a stable liquid form.

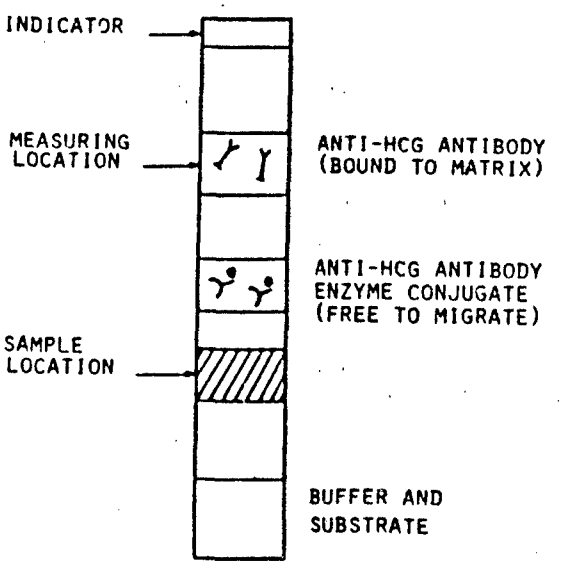


Figure 8. HCG (human chorionic gonadatropine) test strip.

Mechanism of Action of the HCG Immunostrip. The test for the presence of HCG in urine is performed by spotting an aliquot of urine on the measuring location

followed by application of finger pressure to the reservoir. The released developing solution permeates the paper matrix and migrates along the strip in a regular, controlled manner until it reaches the far end. In doing so the urine sample is picked up, together with the enzyme-labeled antibody conjugate and mixed in the zone traveling behind the solvent front.

Heterogeneous Immunoassay. Figure 9 shows the detailed mechanism of reactions on the strip which take place when HCG is present in the sample. Four stages of the development sequence are shown Figure 9 in the adjacent strip diagrams.

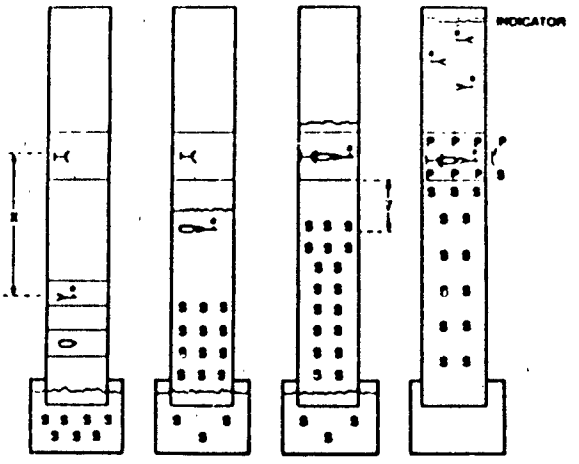


Figure 9. Sequential stages in development of an HCG immunostrip.

The first diagram shows the situation at the zero time point at the initiation of strip development. The distance, x, between the enzyme-antibody conjugate zone and the measuring location is predetermined during manufacture of the immunostrip. Since the migration rate is also largely predetermined by the nature of the developing solution and the strip itself, then the time, t, taken for this solvent front to travel distance x is also predetermined. Time, t, thus represents the reaction time allowed between enzyme-antibody conjugate and HCG present in the sample.

The second diagram shows the formation of an immunocomplex between enzyme-antibody and an HCG molecule. This occurs in a zone immediately behind the moving solvent front. In the third diagram, the solvent front has just passed through the measuring location. Here, the immobilized second antibody specifically binds the HCG enzyme-antibody immunocomplexes, thus trapping enzyme label in the measuring location in direct proportion to the mass of HCG in the urine sample.

The migration continues and, in the fourth diagram, has reached the far end of the strip. This process brings about the separation stage of the immunoassay. Excess enzyme-antibody conjugate, not bound in the measuring location, is carried to the end of the strip and the measuring location itself is washed through with fresh developing solution. The washing stage is extremely effective, with nonspecific binding levels at the measuring location being as low as less than 0.05 percent of the total label.

Differential Migration of Substrate (Cofactor). Baker said that the immunostrip system has been carefully designed to avoid premature reaction of the enzyme-antibody conjugate with the substrate components of the developing solution. If this were not the case then colored product would be formed as soon as the solvent front meets the enzyme-antibody conjugate zone.

Figure 9 depicts the substrate component of the developing solution, shown by an *S* symbol, migrating at a different rate to the solvent front migration. In practice, substrate, or rather cofactor, conversion to a blue product is prevented in the zone immediately behind the solvent front, which also contains the enzyme-antibody conjugate. This is achieved by the fact that the TMB cofactor migrates along the strip with an Rf value of 0.7. Consequently, TMB is absent in the solvent front zone and, although the H_2O_2 substrate is present with the enzyme-antibody conjugate, no blue product can be formed.

The third diagram in Figure 9 indicates a distance, y, between the measuring location and the TMB-containing zone. This distance translates into a volume of developing solution contained over this portion of the strip, and represents the volume of developing solution available to wash nonspecifically bound enzyme-antibody conjugate out of the measuring location.

By the time the strip development is complete, as shown in the fourth diagram, TMB-containing developing solution has passed through the measuring location. As it does so, the blue product is generated in proportion to the amount of enzyme label immobilized in this zone.

Color Development. The product of the TMB-peroxidase reaction is a blue-colored charge transfer complex which binds to the paper matrix in a relatively stable manner and does not migrate significantly. Thus, blue product becomes concentrated at the measuring location, as fresh substrate enters this zone during the final stages of strip development. This phenomenon has the effect of enhancing the sensitivity of signal detection. In the case of an HCG immunostrip, the presence of color at the measuring location would indicate a positive test result and the absence of color a negative test result.

Applications of the Immunostrip System. According to Baker, the versatility of the immunostrip system has been demonstrated for a number of model analytes including bacterial, viral, protein hormone, and steroid hormone targets. It is being developed for applications both for physicians' office testing and home-use testing, primarily for those analytes where a qualitative yes/no result is required, such as the diagnosis of infection or pregnancy. However, with the increasing availability of

portable low-cost reflectance photometers, Baker thinks that a quantitative system is also feasible. Since the immunostrip device is required for nonlaboratory applications, Baker said that it has been designed to be physically and immunochemically robust. The paper strip and the developing solution reservoir are housed in a semirigid plastic casing which forms an effective protective vapor-moisture barrier to the external environment (Figure 10). A tear-off tab reveals the sample application zone, and transparent windows in the plastic casing allow visual inspection of the measuring location and indicator zones. An absorbent pad at the opposite end of the strip to the reservoir serves to improve the wash-through characteristics of the device.

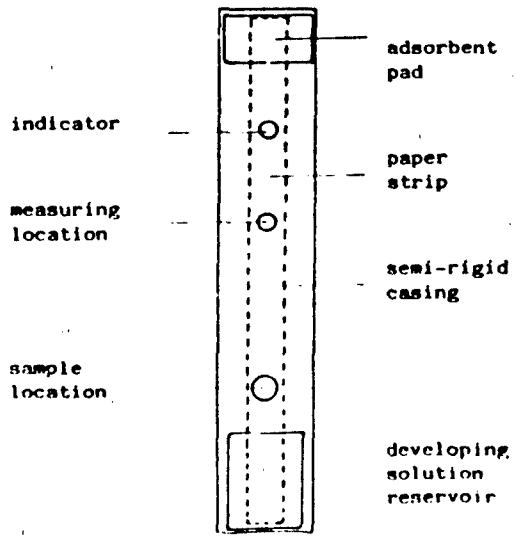


Figure 10. The immunostrip device.

In summary, Baker said that the Immunostrip test is a novel diagnostic system under commercial development. He believes that this system will play an important role in expanding the market in the Alternate Site sector of the diagnostics industry for the following reasons:

1. Ease of use. The immunostrip is a one-step procedure for performing heterogeneous immunoassay. Sample application and initiation by finger pressure is the only operation required.

2. Rapid results. A test result is obtained within the order of 10 minutes. The test is self-timing since a procedural control automatically indicates when the test may be read.

3. Versatility. The immunostrip has been demonstrated to have adequate analytical performance for a range of analytes measured with a variety of immunoassay formats.

4. Convenience of use. The immunostrip can be manufactured as a robust device to give the enclosed reagents enhanced stability and to allow convenience of use in nonlaboratory environments.

ENZYME-AMPLIFIED IMMUNOASSAYS – OPTICAL AND AMPEROMETRIC DETECTION

The technique of enzyme amplification is a method for enhancing the speed and sensitivity of enzyme immunoassays. Essentially the method uses the enzyme label as a "preamplifier" which can activate a secondary enzyme system or "power-amplifier" in order to increase greatly the apparent activity of the enzyme label itself. The enzyme-amplifier can produce either an intensely colored formazan dye or can be coupled to an organic conducting salt or platinum electrode. This topic was discussed by A. Johannsson (Research and Development Division, Novo Biolabs, Cambridge, UK).

Johannsson said that enzyme amplification is a further development of enzyme immunoassays which together with other nonisotopic methods are gradually replacing radioimmunoassays as the methods of choice for the immunochemical determination of analytes of diagnostic significance. Some of the early versions of these assays suffered from poor accuracy and imprecision, according to Johannsson. However, due to rapid technological progress this is no longer the case and most enzyme immunoassays are now superior to their isotopic counterparts in terms of sensitivity, speed, and convenience. Johannsson thinks that advances are also likely to be seen in other important parameters of enzyme immunoassays such as dynamic range and the cost of each determination.

Johannsson said that it has already been shown that the optimal sensitivity of noncompetitive immunoassays is limited by the specific activity of the label employed and its nonspecific signal. As mentioned above, enzyme amplification is a method for increasing the apparent activity of an enzyme label and thus enhancing the speed and sensitivity of enzyme immunoassays. The most commonly used amplifier employs a redox cycle with the reduction of a tetrazolium salt to an intensely colored formazan dye, a product which is measured with a spectrophotometer. This method, according to Johannsson, has found wide application in the field of immunoassay and has been used in the detection of tumor markers and infectious organisms as well as in the measurement of human and animal hormones. Johannsson refers to this as the "optical" enzyme amplification system.

In the redox enzyme-amplifier the enzyme label in the immunoassay, alkaline phosphatase, catalyzes the dephosphorylation of nicotinamide adenine dinucleotide phosphate ($NADP^+$). The product, nicotinamide adenine dinucleotide (NAD^+) then enters a cycle driven by the two enzymes, alcohol dehydrogenase and diaphorase. The tetrazolium salt, INT-violet acts as an acceptor of electrons from NADH and the color intensity

of the formazan dye so formed represents the total charge that has passed through the NAD/NADH cycle (Figure 11).

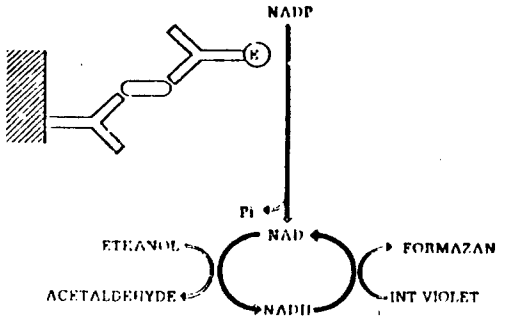


Figure 11. NAD/NADH cycle.

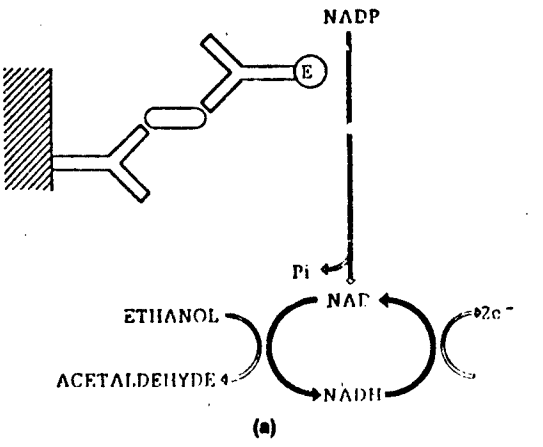
Johannsson also described the development of an "electrochemical" enzyme-amplification system employing amperometric detection of the redox amplifier. This system had been developed in collaboration with scientists at the Cranfield Biotechnology Center, UK. Johannsson said that an organic conducting salt electrode can be used in place of the tetrazolium salt to oxidize NADH directly. Alternatively, the tetrazolium salt can be replaced by the electron carrier potassium ferricyanide, which can mediate the transfer of electrons from the NAD/NADH cycle to a platinum electrode. The principle of these electrochemical enzyme-amplification systems applied to an immunoenzyometric assay is illustrated in Figure 12 (a and b). In 12 (a) the electron flow in the NAD/NADH cycle is coupled to the organic salt electrode. According to Johannsson, the salt that proved to be the most suitable for this purpose was NMP/TCNQ, a combination that had previously been shown to regenerate NAD from NADH directly. However, Johannsson said that he and his group found that the inclusion of diaphorase in the enzyme-amplifier resulted in a higher sensitivity to NADH.

The alternative configuration for the electrochemical enzyme-amplifier, shown in Figure 12 (b), employs ferricyanide as a mediator.

Johannsson also presented some details on the electrochemical enzyme-amplifier system as follows.

Electrochemical Apparatus and Instrumentation.

Current-vs.-time measurements were performed using a two-compartment glass cell with a working volume of 2 ml. In addition to the working electrode, of either platinum or $NMP^+/TCNQ^-$ (N-methyl phenazinium/7,7,8,8-tetracyano-p- quinodomethane), the cell contained a platinum wire counter electrode (3 cm in length and 0.5 mm in diameter) and a saturated calomel reference electrode (SCE). The working compartment of the cell could be mixed during operation with a magnetic stirring bar and reagent additions could be



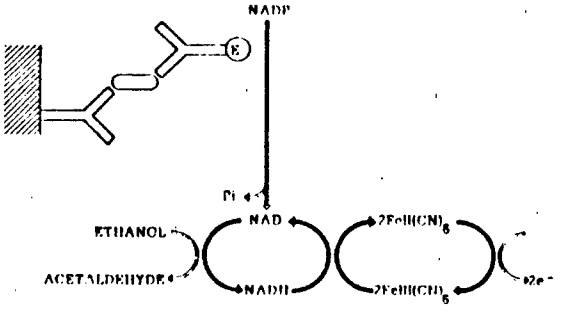


Figure 12. Immunoenzymometric assay.

made directly to the cell via an injection port. Current-time curves were produced with a potentiostat and a y-t flat bed recorder. Current measurements were made at room temperature (22 C.)

Electrochemical Detection of NAD^+

Two kinds of electrodes were used:

1. Platinum working electrode. Prior to use, platinum electrodes were polished with an alumina slurry (0.3-um alumina particles in distilled water). The electrodes were then cleaned in a sonicating water bath to remove the particles and were then subjected to a series of potential steps (-800 mV to + 800 mV) in 0.1 M sodium phosphate, pH 7.0, to remove any surface oxide film. After assembly of the electrochemical cell, 1 ml of 50-mM diethanolamine buffer, pH 9.5, containing 4 percent (v/v) ethanol was added, followed by 1 ml of 25 mM sodium phosphate bu fer containing alcohol dehydrogenase (0.4 mg/ml) and diaphorase (0.4 mg/ml). The contents of the cell were mixed with a magnetic stirring bar and, during the mixing, 0.010 ml of a solution of potassium ferricyanide was added to give a final concentration of 12.5 mM, followed by an aliquot of a solution of NAD^+ in the diethanolamine buffer described above. A potential of + 450 mV (vs. SCE) was applied to the working electrode

immediately after the addition of NAD, and the current was measured for a further 20 minutes; the cell contents were stirred throughout the measurement period. After each measurement the electrochemical cell was dismantled and the platinum working electrode was rinsed with distilled water and dried before reassembly.

2. Organic conducting salt working electrodes. The salt of $TCNQ^{\cdot}$ and NMP^{+} was prepared according to a previously described method of L.R. Melby. The working electrode was prepared by applying a drop of a solution of the salt, in tetrahydrofuran, to the surface of a carbon foil disc of 3 mm diameter. After evaporation of the solvent the carbon disc was bonded to a platinum wire using silver-loaded epoxy adhesive. The electrochemical measurements were made essentially as described above, except that ferricyanide was not present in the cell and, while the cell contents were stirred during the addition of NAD^{+}, the current measurements were made 1 minute after the stirring was stopped. In addition, a lower potential of +250 mV (vs. SCE) was applied to the working electrode.

Optical Enzyme Amplification

The dephosphorylation of NADP by alkaline phosphatase occurs at a rate of approximately 80,000 molecules per minute per molecule of enzyme, according to Johannsson. This is slightly slower than the rate of color formation from para-nitrophenylphosphate and other commonly used substrates for enzyme immunoassays. However, each molecule of NAD^{+} generates between 30 and 300 intensely colored formazan molecules every minute dependent on the concentration of the amplifying enzymes, alcohol dehydrogenase and diaphorase. Also the "power" amplifier has a near linear response to the product of the "pre-amplification" step, NAD^{+}.

Johannsson said that the sensitivity of such a system is sufficient to permit the detection of 0.01 of an attomole, or 6,000 molecules of alkaline phosphatase. The application of enzyme-amplification to a "sandwich" or excess labeled reagent immunoassay demonstrated a detection limit of about 280,000 molecules of antigen (in this case, human thyroid-stimulating hormone). The sensitivity of this immunoassay was found to be limited largely by the relative error of the nonspecific signal from the labeled reagent (rather than by the specific activity of the detection system itself), thereby demonstrating that enzyme-amplification fulfills the requirements for an ultrasensitive nonisotopic detection system, according to Johannsson.

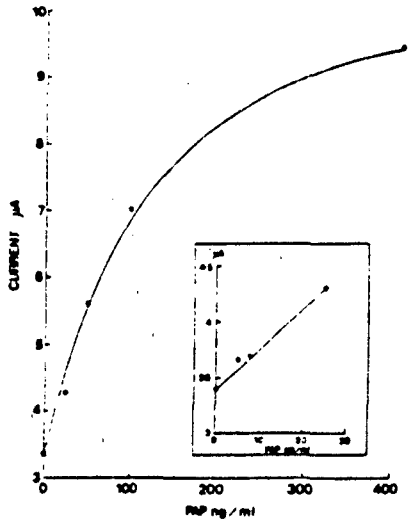
Johannsson said that a limitation of all optical detection systems is the relatively narrow dynamic range which can be obtained owing to the difficulty in measuring – with sufficient accuracy and precision – absorbance values that differ by more than two orders of magnitude. Figure 13 (a), which shows an optical assay for prostatic acid phosphatase (PAP) illustrates the relatively narrow measurement range. This can be partly remedied, according to Johannsson, by monitoring the rate of color development over a period of time and relating the rate of color development to the analyte concentration. However, the measurement of current or charge is not expected to suffer from the same limitation.

Electrochemical Immunoassays

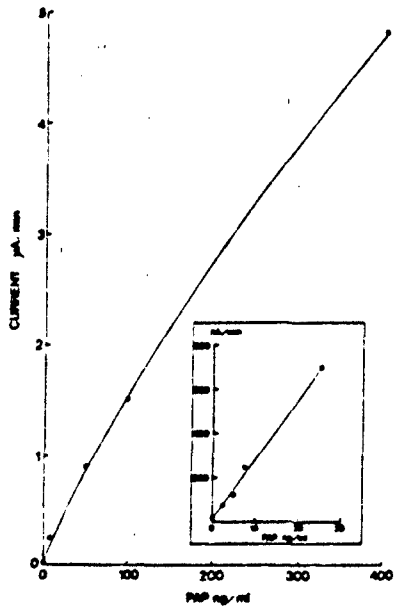
The optical and electrochemical methods were compared by Johannsson and his group in an immunoenzymometric assay for human PAP, where the immunoassay procedures were identical up to and including the dephosphorylation of NADP by the enzyme label, alkaline phosphatase. In the optical methods, an enzyme-amplifier containing INT-violet was added and the color intensity was measured at 492 nm (Figure 13 (a)). In the electrochemical methods a cell containing the appropriate working electrode, a platinum counter and a silver/silver chloride reference was used to measure the NAD generated by the enzyme label, Figure 13 (a) and (b). Detection limits for PAP were determined for all three measurement methods and were: optical, 0.25 ng/ml; electrochemical (ferricyanide), 0.27 ng/ml; and electrochemical ($NMP^{+}/TCNQ^{\cdot}$), 0.61 ng/ml. Thus the electrochemical methods were of comparable sensitivity to that achieved with the existing optical system. However, the electrochemical methods demonstrated considerable advantage in dynamic range where, owing to the inherent limitation of optical measurement, the highest concentration of PAP that could be used in the calibration curve was 7.5 ng/ml, whereas in the electrochemical systems it was greater than 400 ng/ml.

Johannsson said that the sensitivity of both electrochemical detection systems was greatly increased by the inclusion of diaphorase, which, therefore, is assumed to catalyze the transfer of electrons from $NADH^{+}$ to the $NMP^{+}/TCNQ^{\cdot}$ electrode and from $NADH^{+}$ to ferricyanide. Although Johannsson believes that the detection limit for cofactor can be further improved, the value obtained for the potassium/ferricyanide system is about two orders of magnitude better than previously reported for $NAD^{\cdot}$ with an alcohol dehydrogenase/NMP^{+} electrode.

According to Johansson, a further advantage in the adoption of electrochemical methods for detecting enzyme labels in enzyme-amplified immunoassays means that the cost and complexity of the measuring instrument can be substantially reduced, thereby making such assays more attractive to the smaller laboratory. A future development in this area is the use of electrode arrays for the measurement of the multiple tests carried out in microtitration plate wells. Johansson and his group have also shown that amperometric measurements of enzyme amplifiers can be carried out successfully in unstirred solutions which may lead ultimately to the development of a sensitive, convenient and cheap detection system applicable to most immunoassay configurations.



(a)



(b)

Figure 13. Optical assay for prostatic acid phosphatase (PAP).

RAPID MICROBIAL ASSAY TECHNOLOGY

This topic was addressed by A. Swain (Biotechnology Center, Cranfield Institute of Technology, UK). Swain said that traditionally assessment of the microbiological status of a given sample has involved the standard plate count, enabling an estimate of the total number of viable cells in the sample plated out, by counting the number of bacterial colonies which develop following incubation. Frequently the time required for the organisms to grow sufficiently to form visible colonies is 48 to 72 hours. Such time scales are undesirable to manufacturers paying out storage costs on products awaiting clearance for dispatch, or to clinicians who need to prescribe appropriate medication.

According to Swain, there is, therefore, an increasing trend towards the development and implementation of rapid methods for detecting microbial contamination. For example, urine cultures are the most common type of specimen processed in the diagnostic microbiology laboratory; about two-thirds of these prove bacteriologically negative, Swain said. Therefore, it is obviously advantageous to eliminate these negative specimens rapidly. Food microbiology also has a particular need for rapid methods which may enable food processors to more readily reject unacceptably contaminated raw foodstuffs prior to processing, to monitor the processing lines for early evidence of microbial problems, and to improve the quality of the final product while reducing costly inventories as well. According to Swain, an estimated saving of $1.8 billion per year may be achievable in the UK alone by the introduction of appropriate rapid microbiological assay technology.

Swain's presentation was concerned primarily with the detection and enumeration of microorganisms, rather than with the identification of specific strains or species. He said that the term "rapid method" refers in general to any technique which allows significant time savings when compared with traditional methodologies – i.e., generally speaking, a method taking less than 24 hours – although, of course, the ultimate aim must always be the attainment of real-time analysis. According to Swain, the rapid methods available can be categorized according to the particular mode of detection exploited – for example, biophysical, biochemical, bioelectrochemical, etc., and, of course, according to the requirement of the end user. For example, busy clinical microbiology laboratories have a requirement for automated multichannel devices designed to handle high sample throughput, whereas environmental microbiologists or general practioners want desk-top, portable, single-test instruments requiring a minimum of sample manipulation/pretreatment and producing a result in minutes rather than hours.

Biochemical Methods

Reactions which produce light (bioluminescence and chemiluminescence) can be used to measure as little as a picogram (10^{-12} g) of analyte such as ATP, NADH, and many organic and inorganic compounds. Swain said that there are several instruments available on the market which harness these reactions for the estimation of biomass. The Lumac Biocounter (Lumac b.v., Schaesberg, the Netherlands) detects bacterial ATP via bioluminescence. The light produced as a result of a

reaction catalyzed by firefly luciferase is detected photometrically:

$$ATP + Luciferin + O_2 \rightarrow Oxyluciferin + AMP + PPi + CO_2 + light$$

The amount of light produced, measured by a luminometer, is proportional to the amount of ATP present in the sample. The bioluminescence assay is based upon the assumption that living cells of a given type contain a reasonably constant amount of ATP, thereby enabling correlation of ATP concentration with the number of microbial cells present. The Biocounter, according to Swain, is capable of detecting 10^3 colony-forming units per ml, the results being digitally displayed in 10 minutes to 2 hours depending upon the sample. Lumac has designed pretreatment protocols for samples containing both microbial and nonmicrobial ATP to detect, for example, the presence of somatic cells in urine or in milk. The somatic cells are differentially lysed and the ATP released is destroyed by the addition of ATPase.

Swain said that, as is the case with the majority of rapid methods available, careful optimization of the approach has to be made according to the sample type being examined – for example, in order to minimize light quenching by the extraction chemicals, buffers, or other substances in the sample.

According to Swain, instruments have recently become available for monitoring fermenter biomass concentration by the measurement of NADH-dependent fluorescence (Fluoromeasure, Ingold Electrodes Inc., Wilmington, Massachusetts; Fluoromeasure NADH Detector, BioChem Technology, Inc., Malvern, Pennsylvania). These on-line systems have been used to provide real-time biomass concentration data for specific fermentations – having first obtained cell density-fluorescence correlation factors over a series of prior fermentations. The relationship is very dependent, according to Swain, on the culture environment; for example, the presence of medium constituents which fluoresce can cause problems.

Catalase is an enzyme found in many aerobic bacteria. The enzyme breaks down hydrogen peroxide into hydrogen and water. Because catalase activity increases with the bacterial population it can be used to estimate the bacterial concentration in certain products. Swain mentioned the Catalasemeter (Bio-Engineering Group Ltd., New Haven, Connecticut) which uses the principle of disc flotation to quantitatively measure catalase activity in foods. The meter is capable of detecting 10^4 bacterial cells/gram of sample in around 30 minutes. A culture assay paper disc is inserted into a hydrogen peroxide-containing test tube in the Catalasemeter. In the presence of catalase, gas bubbles form and are entrapped beneath the disc, causing it to float. The time taken for the disc to float up after sinking is designated as its flotation time, which is inversely proportional to the concentration of catalase in the test liquid. According to Swain, this instrument is particularly useful for detecting contamination in cold stored food since in these conditions, around 95 percent of the relevant bacteria are catalase positive (pseudomonads, micrococci, etc.). One problem, however, is the inability to distinguish between catalase of bacterial and nonbacterial origin.

Biophysical Methods

The direct epifluorescent filter technique (DEFT) was developed by the National Institute for Research In Dairying, UK. The technique involves pretreating the sample with enzyme and detergent, filtering it through a membrane, and then staining the retained bacteria with Acridine Orange prior to counting with a fluorescence microscope. Generally, viable "active" organisms stain orange whereas the nonviable ones stain green. Swain said that problems due to operator fatigue encouraged the development of a semiautomated system which incorporates an image analyzer for automatic counting and an improved reagent system and filtration unit. The Bio-Foss Automated Microbiology System (Foss Electric Ltd, York, UK) is based on the DEFT principle. It is mainly used to detect bacteria in samples from the food and drink industry, the published limits of detection being 5×10^3 to 5×10^8 bacteria/ml.

The Autotrak (A.M. Systems, Glasgow, UK) has been developed in the Center for Bio-Medical Instrumentations, University of Strathclyde. It is also based on DEFT. This system is essentially a microprocessor-controlled epifluorescence microscope equipped with a flow-through stage. Liquid samples are taken up on a probe and applied to a specially treated moving tape. The samples on the tape are then fixed and stained by a fluorochrome before passing the microscope objective. A result is achieved within 60 seconds of sample input to the system and the throughput can be as high as 120 samples per hour. The Autotrak system has been applied to the routine screening of urine specimens and showed high sensitivity and specificity. However, according to Swain, this technique is not problem free. Interference from particulate matter can occur and there may be the requirement for a preincubation period of up to 72 hours to allow for the recovery of stressed organisms in the sample.

Another example of a direct counting system is the Orbec Ramus 256 (rapid, automatic, microbiological, urine screening), developed by Orbec Limited, Sanderstead, Surrey, UK. This is a direct electronic counting system which monitors the effect of microorganisms on an electric field as they traverse that field. As the relatively nonconducting organism passes through the field, the electrical resistance within the aperature is increased, giving rise to a transient drop in voltage. The assumption is that the magnitude of the pulse is proportional to the size of the microorganism, such that particles giving pulses of a given magnitude can be counted. Swain said that it is essential that such an instrument be used on a sample of optimized concentration,

since large concentrations increase the occurrence of coin drawback with this system is that it produces a whole cell count and not a viable count, since it cannot distinguish between nonviable and viable cells. The Orbec system is used to test samples for the presence of "significant" numbers of bacteria (10^5/ml) and leukocytes (10/μl), and is capable of analyzing 100 samples/hr.

A new instrument for the real-time estimation of biomass in fermenters, the "Bugmeter" (Dulas Engineering Ltd, Powys, UK) is based on the principle of dielectric permittivity. The dielectric permittivity of a suspension of microorganisms is very much greater than that of the suspending medium or of noncelluiar particles, due to the phenomenon of dielectric dispersion (Harris et al., 1987). The permittivity (capitance) of cell suspensions at low radio frequencies is linear with the volume fraction of the suspended phase (biomass) up to very high concentrations. The Bugmeter is therefore based on a small radio frequency capitance probe which is inserted into the fermenter broth, providing a means of rapid detection for noninvasive biomass determination. The lower limits of detection for this instrument will vary depending on the bacterial cell volume; i.e., the smaller the cell volume, the larger the number of bacteria needed to reach the detection threshold. Despite this limitation, the Bugmeter appears to satisfy a long-term requirement for real-time biomass monitoring in fermenters.

The Bac-T-Screen (Marion Laboratories Inc., Kansas City, Missouri) is a colorimetric filtration method developed for urine screening. One ml of well-mixed urine is suctioned through a piece of filter paper attached to a card. The urine diluent (3 ml) is drawn through the filter, followed by mixing with 3 ml of safranin dye and a final rinse with a decolorizer. A positive urine has a pink color on the filter card of 1+ intensity; this is indicative of a urine specimen containing 10^5 cfu/ml. The entire test takes only 2 minutes. In trials, a small proportion of samples proved uninterpretable for one of two reasons. Either the sample clogged the filter or it left a residual pigment. The former was generally found to be due to the presence of white blood cells, and these tended, on plating, to give positive results for bacteria. The pigmented samples, however, generally produced negative bacterial counts. Swain said that the overall appeal of this method was the advantage of its being a 2-minute test.

All of the biophysical methods described above can be considered to be direct, since they, by a variety of means, detect the numbers of organisms in a sample at a given moment in time. There also exist a variety of methodologies which are dependent on cell growth. This consequently increases the timescales involved in obtaining results, but at the same time enables the limits of detection to be broadened by allowing bacteria to multiply to a detectable threshold level.

Impedence and conductance measurements can be used to assess microbial populations. As microorganisms grow they utilize nutrients in the medium, converting them into smaller, highly charged molecules – for example, fatty acids, amino acids, and various organic acids. When electrodes are immersed in the medium and an alternating current is applied, the flow of current changes as a result of the metabolic activity of the microorganisms. Typically, impedence decreases while conductance and capitance increase. When the microorganisms reach a certain threshold (for example, 10^6 to 10^7 bacterial cells/ml, 10^4 to 10^5 yeast cells/ml), the impedence changes markedly. The time taken for an incubated sample containing even one microorganism to reach the threshold is reported as the impedence detection time. This can then be used to estimate the initial concentration in the sample. In general, the higher the initial concentration of microorganisms, the faster their detection time.

The Bactometer Microbial Monitoring System manufactured by Bactomatic Ltd. (Henley-on-Thames, Oxon, UK) utilizes the impedence principle to enumerate microbial populations. The fully automated, computer-controlled system monitors and plots an impedence curve which closely resembles a standard bacterial growth curve. The instrument can selectively measure total impedence or either of its two components, conductance and capitance, thereby offering flexibility in the range of applications possible. The Bactometer is widely used in the food, drink, and cosmetics industries. The instrument is capable of completing sterility tests within 24 to 48 hours.

The Malthus-AT (Malthus Instruments, Stoke-on-Trent, UK) uses conductance monitoring to estimate microbial population. This system, too, is fully automated and computer controlled once the sample has been loaded.

One of the major factors influencing the quality of data obtained by these instruments, according to Swain, is the method of sample preparation. These systems can prove very effective once experimental protocols have been devised for the particular sample type under test.

One of the most obvious changes which occurs during the growth of all microorganisms is the production of heat. The heat produced can be measured by very sensitive microcalorimeters, according to Swain. Microcalorimetry has been used to enumerate microorganisms in foods. The procedure involves correlating a thermogram which shows the pattern of heat generation during microbial growth, with the absolute number of microbial cells. The Thermal Activity Monitor (LKB Instruments, Croydon, Surrey, UK) is a four-channel microcalorimeter which can be used to monitor microbial growth in such samples as milk, meat, molasses, and canned foods.

Radiometric procedures can be used to detect viable microorganisms cultured in media containing isotopically labeled substrates, by measuring the radioactivity associated with their metabolites. Results for sterility testing of aseptically packaged products can be obtained

in 4 to 5 days as opposed to over 10 days by conventional methods; the enumeration of microorganisms in food samples can be achieved in under 24 hours. Bactec (Johnston Laboratories, Towson, Maryland) is a commercially available automated system which uses the principle of radiometry. It has been found that 10^7 cells can be detected in 1.5 to 2.5 hours. Also 6 to 7 hours was found to be a time sufficient to differentiate suspect from noncontaminated food samples using radiometric procedures.

There are several instruments available which are based on the photometric detection of growth – for example, the AutoMicrobic System (AMS Vilek Systems, Inc.), the Autobac (General Diagnostics, Warner-Lambert Co., Morris Plains, New Jersey), and the Bioscreen (Labsystems (UK) Ltd., Uxbridge, UK). Such systems possess a photometer module which detects changes in lightscatter as a result of bacterial growth. As the bacteria grow, the turbidity of the broth increases, which in turn causes an increase in lightscatter and a drop in voltage. A voltage change of 0.2 units within 6 hours indicates a positive urine when the Autobac system is used. Such photometric devices have proved useful, according to Swain, particularly in the field of urine screening/contaminant identification.

Bioelectrochemical Methods.

The Biotechnology Center at Cranfield is involved in the research and development of a wide range of amperometric biosensors. The biosensor configurations which have received the greatest attention are electrochemical transducers used in conjunction with enzymes (Cardosi and Turner, 1987), the biological component and the electrode being coupled via suitable artificial electron mediators. An amperometric biosensor has been developed at Cranfield for the prediction of the shelf-life of chilled meat joints. By measuring the concentration of marker chemicals (in this case, glucose), an indication of the microbial status of the foodstuff is obtained. This is so because of the preferential consumption of glucose by the microbial flora at the surface of chilled fresh meat, causing the establishment of a glucose concentration gradient from the surface into the depth of the meat as the flora increases. According to Swain, the biosensor is composed of four glucose electrodes, which enable the measurement of glucose concentration at various depths in the meat producing a glucose profile from which the shelf-life prediction is made (Kress-Rogers and D'Costa, 1986). The planar glucose sensor array is designed to be mounted in a knife-type instrument which, with the help of a cutting edge, can be inserted into meat joints. A new electrode configuration has been developed to improve the electrode performance at the low temperatures (2° to 10° C) and pH values (around 5.5) encountered in chilled meat.

Swain said that in the course of the last 3 years a bioelectrochemical method for the assessment of microbial activity has been developed at Cranfield under the sponsorship of Paul de la Pena Ltd. and the Department of Trade and Industry, UK. This work has resulted in the production of a prototype instrument called "The Biocheck." Its design specifications are significantly different from the many instruments and methodologies described earlier, according to Swain. These specifications are:

- Rapidity of operation – 5 to 10 minutes to obtain a result
- Portable, hand-held and battery operated
- Inexpensive – basic hardware, using disposable materials, for each test
- Accurate to discrete orders of magnitude of bacterial contamination
- Robust and easy to use by unskilled personnel
- Minimum of sample handling – a preconcentration step only being necessary for lower bacterial contamination.

The basis of the technique concerns the use of a proprietory cocktail of chemical mediators which are used to divert electrons from the respiratory chain of microorganisms to an amperometric detector, thereby diverting them from the natural electron acceptor. The mediators are reduced by electrons from the electron transport chain and are subsequently reoxidized at an electrode which is held at a constant preset potential. The current thus produced during a 2-minute test period is proportional to the number of bacteria present in the stirred solution and to their metabolic activity.

The Biocheck was envisaged as a means of assessing gross contamination comprised of mixed populations of microorganisms, according to Swain, who was directly involved in the development of the Biocheck. He said that in order to satisfy this requirement, maximum effort was spent on ways of optimizing the reponse from a wide range of bacterial genera. Therefore, Swain and his group embarked on a program of screening large mediators. Unlike the majority of biosensor configurations, where the ability to immobilize the mediator is essential, this particular amperometric technique relies on the mediator being sufficently water soluble to shuttle electrons from the bacteria to the electrode. Early work showed, according to Swain, that potassium ferricyanide gave the highest response with the majority of the bacteria tested, and coupled with the fact that it was not autooxidizable, this mediator appeared promising. Unfortunately, one drawback was the poor response obtained with pseudomonads, since this genus is highly relevant in a wide range of industrial and food samples. Further optimization of the mediator used in the bioelectrochemical response was necessary therefore, and a number of different mediators were considered, including a range of ferrocene derivatives, thionine, and various quinones. According to Swain, the breakthrough came with the combination of ferricyanide with p-benzoquinone into a mediator cocktail (Turner et al., 1987). This cocktail, for

example, increased the response of *Pseudomonas aeruginosa* by over 100-fold compared to ferricyanide alone, and, more generally, the use of this cocktail made it feasible, according to Swain, to claim the distinction between orders of magnitude of mixed microbial populations. Table 2 shows the range of types of cultures detectable using the mediator cocktail.

Table 2. Microorganisms detected bioelectrochemically.

Achromobacter album	*Lactobacillus plantarum*
Acinetobacter calcoaceticus	*Micrococcus lysodeikticus*
Agrobacterium tumefaciens	*Mycobacterium smegmatis*
Alcaligenes faecalis	*Nocardia* (NCIB 8863)
Bacillus licheniformis	*Proteus vulgaris*
Bacillus megaterium	*Pseudomonas aeruginosa*
Cladosporium resinae	*Pseudomonas fluroescens*
Enterobacter aerogenes	*Saccharomyces cerevisiae*
Enterobacter cloacae	*Salmonella typhimurium*
Escherichia coli	*Staphylococcus aureus*
Klebsiella aerogenes	*Streptococcus faecalis*
Lactobacillus bulgaricus	

Swain said that in addition to mediator optimization it was essential to optimize the working electrode materials. In particular, high-surface-area carbon and graphite materials were examined, in preference to noble metal working electrodes since a one-use-disposable BEC configuration was required. The high-surface-area electrodes resulted in limits of detection of 10^5 cfu/ml.

The prototype development using the benzoquinone/ferricyanide mediator cocktail with a disposable BEC based on a glass vial incorporating freeze-dried reagents (phosphate buffer, KCl, and glucose) with a molded plastic holder retaining a graphite working electrode and an Ag/AgCl reference electrode (see Figure 14) has been described in detail (Turner et al., 1987).

Swain said that in order to carry out the amperometric test method the sample must be introduced into the vial of freeze-dried reagents in a liquid form. Location of the BEC in the Biocheck completes an electrical circuit which results in the software turning on the stirring and applying the potential. After a settling down period, generally less than 5 minutes depending on the sample type, an increase in current can be monitored when a sufficiently high number of bacteria are present. The change in current over a 2-minute period is measured and at this stage of development the maximum slope, in μA/min, is shown at the end of the test. The instrument has been used, according to Swain, to examine a wide range of real samples and Table 3 shows that it has been possible to detect a wide range of bacteria used to spike such samples.

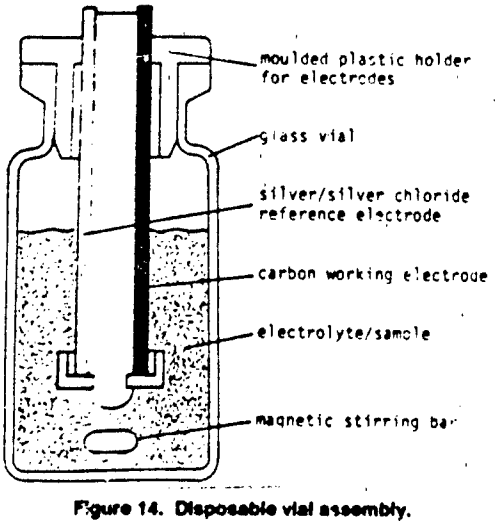


Figure 14. Disposable vial assembly.

Table 3. Samples tested in the biocheck.

Sample	Bacterium detected
Sterilized milk	*Streptococcus faecalis*
Raw milk	*Escherichia coli*
Raw milk	*Pseudomonas aeruginosa*
Raw milk	*Lactobacillus bulgaricus*
Cooling water	*Enterobacter cloacae*
Colling water	*Pseudomonas aeruginosa*
Meat washings	*Salmonella typhimurium*
Beer	*Escherichia coli*
Swimming pool water	*Klebsiella aerogenes*
Cutting fluid	*Escherichia coli*
Vegetable washings	*Escherichia coli*

Swain said that a good correlation between the viable counts and the Biocheck reading has been obtained with *E. coli* at room temperature using a disposable BEC based on elements manufactured on a large scale (electrodes, holders, and vials of freeze-dried reagents).

At present an in-house field trial is underway, according to Swain, monitoring the level of microbial activity from sewage from a local sewage works. This real sample provides a supply of viable organisms in the range of 10^5 to 10^7 cfu/ml. Samples are taken from both the inlet and outlet stages of the works. Swain said that it has proved necessary to incorporate a prefiltration/preconcentration step, particularly for the inlet samples which display electrochemical interference due to the high level of organic matter present; although it is possible to detect the bacteria in raw samples directly (by comparison with a heat-treated control). On this basis it is possible to obtain a positive reponse for as low as 2×10^5 cfu/ml in the outlet sample. Swain showed a calibration plot of log viable counts against log Biocheck reading for monitoring sewage. A positive correlation with a regression coefficient of 0.9418, which related to about 30-percent error in the average readings, was achieved.

According to Swain, these results confirm the use of Biocheck for the detection of a mixture of bacteria in a real sample, with the ability to distinguish across at least three orders of magnitude down to low 10^5 cfu/ml. Swain said that it is hoped that the Biocheck can be applied to a wide range of sample types, incorporating where necessary a simple pretreatment step which should essentially appear user invisible. The speed of operation, simplicity,

and portability of the Biocheck assay assures its place alongside the vast range of expensive laboratory instruments designed to satisfy the undoubted requirement for rapid microbial assay technology.

DIAGNOSTIC APPLICATIONS OF DNA PROBE TECHNOLOGY

This topic was discussed by T.M. Twose (ICI Diagnostics, Cheshire, UK). Twose thinks that DNA will join the ranks of routine diagnostic analytes, alongside proteins, enzymes, metabolites and drugs. However, with the technology still in its infancy, it is difficult to predict with certainty how and to what extent it will be used.

Twose said that DNA has several technical advantages as an analyte which can be exploited in different combinations depending on the application. Fundamentally, the information contained in its linear sequence has unique value. Otherwise, DNA is chemically uniform and it is robust, stable, and ubiquitous, providing important benefits in sample preparation. Twose said that the applications are clear – for example, detection of pathogens, genetic disease diagnosis, tissue typing, paternity and forensic tests, cancer diagnosis, and risk/predisposition testing for common diseases.

DNA probes are generally short lengths of DNA, used in single stranded form. Incubated under appropriate conditions with the separated strands of a target DNA molecule, a double helical structure is formed. By virtue of the base-pairing interaction this binding is specific for the complementary sequence. The hybridization reaction is rapid and binding is tight. DNA probes are used to detect DNA (and RNA) of diagnostic interest at various levels of specificity (genus, species, etc.), for instance, in pathogen detection tests. The fidelity of base-pairing interactions can be exploited though to a very high degree of specificity for fine structure and sequence analysis; the limit is being able to detect one nucleotide difference in the human genome. Twose said that from a manufacturing standpoint, DNA probes are well-defined chemically, are stable, and for many applications can be synthesized automatically on a machine, reliably and at low cost. However, to realize these fundamental advantages to meet the user's needs for sensitivity, speed, convenience, and cost effectiveness across a wide range of applications, numerous technical obstacles need to be overcome.

According to Twose, sensitivity is a major hurdle, with many applications providing less than 10^4 molecules of analyte (i.e., less than 10^{-20} moles). Approaches to this include use of abundant – but less ubiquitous – targets (ribosomal RNA), enzymatic amplification of the target, probe, and enzyme networks, and amplification of the signal.

The properties inherent in DNA (for example, ability to copy/replicate using polymerases) open up a number of approaches to assay design which are not applicable in immunoassays, according to Twose. Initially the province of the molecular geneticist and the nucleic acid chemist, increasingly, skills from "mainstream" diagnostics industry disciplines are being applied as the technology moves out of the research laboratory. Twose said that this is of critical importance in solving problems in sample preparation, format, signal/detection and automation--problems which are interrelated and which will be solved only by a concerted systems approach. Technological progress will speed up now as transferrable know-how, especially from immunoassay systems development, becomes more readily applicable.

Sufficient progress has been made for the first diagnostic products to be launched in the form of kits. Although they can be improved upon, they represent a substantial addition to our diagnostic capabilities, according to Twose. Eleven kits for pathogen detection have received FDA approval in the US, nine of them to the US company Gen Probe. More advance in commercial terms, according to Twose, is the research-use market with several companies (Amersham, DuPont, Oncor, Life Technologies and others) actively offering products. However, at the present time, revenues overall from the sale of gene probe-base products are modest, probably not exceeding $10 million.

Other companies have set up reference laboratories particularly for human genetic applications, where methodologies are still technically demanding. In the US, Integrated Genetics and Collaborative Research are offering a range of genetic disease tests based on RFLP technology. Oncor, Gen Care and others offer services to clinical research, especially cancer. Lifecodes in the US and ICI's Cellmark Diagnostics in both the US and Europe have targeted the higher volume service sector of paternity and forensics testing first with clinical diagnostics as a natural next step.

Twose said that rapid progress is being made in human genetics, an application area which gene probes will dominate. Clinically important single gene defects have been identified. Understanding of the molecular genetics of cancer is advancing well on several fronts, according to Twose. The genetic contribution to common disorders such as atherosclerosis, autoimmune disease, and CNS conditions is beginning to be understood. Full maps of the human genome are becoming available, with the first map at about 10 centimorgans (10^7 base pairs) resolution having been announced recently. Twose said also that new, highly polymorphic probes based on VNTR's (variable number of tandem repeats) are making a key contribution. Forensic identification of blood, hair, and semen and other tissues is being revolutionized by this new technology, particularly by the minisatellite probes discovered by Jeffreys in the UK.

Twose thinks that, controversial though it is, if the total sequencing of the human genome goes ahead it will provide a strong boost to human genetic diagnosis in the long term. High-speed automated sequencers will be further refined and may be operated on a commercial basis

in centralized facilities as a service, according to Twose. The shift of sequencing from a research environment to a production environment should improve cost-effectiveness both for research support and diagnostics.

Twose said that earlier projections of sales can now be seen to have been optimistic. Products have been slower coming to the market than had been predicted. Commercial activity is picking up with a small number of companies taking the lead in addressing various segments of the market while others are waiting to see how the field develops commercially.

There are applications where gene probe technology is uniquely suited, and where there are unsatisfied user needs as, for example, in genetic disease testing and risk/prediction testing. Twose said that apart from technological challenges, these particular applications present educational and resource challenges, and legal, social, and ethical issues need to be resolved. Gene probe technology will be dominant, he said, will open up new possibilities, but that the market will develop slowly.

In other areas, pathogen detection, for example, products based on gene probes will open up, according to Twose. However, they will have to fight it out with other technologies for the higher volume mainstream markets. Performance, cost, convenience and automation will be the key factors. Technological progress towards these objectives is rapid and shows no signs of slowing down. Twose thinks that there are no reasons to believe that the remaining hurdles cannot be surmounted so that competitive products can be launched. To replace culture-based techniques, antibiotic resistance/sensitivity information will have to be provided, but this appears feasible using gene probes, according to Twose. He said that taking a longer term view the commercial future of gene probes for pathogen detection is bright.

DNA PROBE TECHNOLOGY–IN SITU HYBRIDIZATION ASSAYS

This topic was addressed by N. Kelker (Enzo Biochem, New York, US). Kelker said that DNA probes may be used to identify organisms or genetic traits based on the presence of specific chromosomal DNA or RNA sequences. Novel nonradioactive methods for detecting the hybridization of DNA probes to these specific sequences have recently been developed. These methods permit hybridization events to be monitored visually by color or fluorescence. The marriage of these nonradioactive labeling methods to DNA probe assays has led to the development of a variety of rapid, sensitive, and specific diagnostic products for identifying bacterial and viral DNA's directly in cells by *in situ* hybridization. The tests do not require the use of radioactive materials or highly specialized equipment. The result is that reliable diagnoses of illnesses that have historically been difficult to diagnose can now be made cheaply, with a minimum of effort, and often in less than 1 day. Lengthy culture times can be reduced or eliminated. The development of nonradioactive *in situ* hybridization assays for the detection of several viral pathogens, such as human papilloma virus (HPV), herpes simplex virus (HSV), and cytomegalovirus (CMV) were described by Kelker.

Nonradiactive In Situ DNA Hybridization Assays

In situ DNA hybridization assays involve the hybridization of DNA probes to complementary sequences (i.e., target DNA sequences in the host organism or in the chromosomes of an invading viral or bacterial pathogen) directly in the cell. Hybridization of labeled DNA probes to the target DNA sequences and detection of these hybridized probes are performed on fixed cells or tissue sections which are attached to a microscope slide. According to Kelker, this DNA hybridization assay format allows genetic information to be obtained simultaneously with cytologic and histologic features. Thus, *in situ* hybridization assays represent an additional "window" into the cell, which ultimately permits more sensitive and accurate diagnoses to be made. Kelker said that these assays are particularly useful for the detection of viral infections involving a small subset of cells in the test specimen – i.e., cases in which only one cell out of every 10^5 or 10^6 cells in a test specimen are actually infected with a virus. Southern blot or dot blot hybridization assay formats would be less likely than *in situ* hybridization assays to detect viral DNA sequences under these circumstances, since the *in situ* tests can detect a single virus-infected cell, according to Kelker.

Kelker said that the nonradioactive DNA detection systems developed by him and his group have facilitated the application of the *in situ* hybridization technology to clinical diagnostics. Radioactively labeled DNA probes can and have been used for *in situ* hybridization assays in research laboratories, but these assays are lengthy, cumbersome, and often insensitive, according to Kelker. Weeks are often required before the results of these assays can be determined by autoradiography. The analysis of these radioactive assays is also tedious and error-prone. They are not suitable for use in clinical laboratories.

In contrast, fluorescence and enzyme-linked color detection systems allow hybridization events to be monitored visually using a simple light or fluorescence microscope. Kelker said that highly trained personnel or sophisticated machinery are not required for either the performance or the interpretation of these tests. In addition, these assays are quite rapid and accurate assay results can be obtained in 1 hour or less, according to Kelker.

The enzyme-linked, color-generating, hybridization-detection systems developed by Kelker and coworkers for use in DNA probe assays is based on the findings of D. Ward and his colleagues at Yale University. Labeled DNA probes are prepared by incorporating biotin, in the form of the biotinylated nucleotide Bio-11-dUTP, into the DNA structure. The binding of another protein,

either avidin or streptavidin, to the biotin moiety of these biotinylated DNA probes can be monitored using avidin-enzyme (or streptavidin-enzyme) complexes. The complexed enzyme is selected for its ability to catalyze the production of colored products upon reaction with colorless substrate molecules in the presence of a chromagen. Quantitation of bound, or hybridized, probe DNA can be made on the basis of this color reaction. Horseradish peroxidase (HRP), alkaline phosphatase, and acid phosphatase are commonly used for this purpose.

Kelker presented the protocol used to perform *in situ* DNA hybridization assays using this enzyme-linked detection system as follows. First, the sample to be examined (a patient specimen or innoculated cultured cells) is attached to a standard microscope slide, then fixed with appropriate reagent. The DNA probe, which has already been labeled with biotin, is added. The slide is then placed onto a heating block for 2 to 3 minutes to allow both the probe and the target DNA's to be separated from their complementary strands (denatured). Hybridization is allowed to proceed for 10 to 30 minutes. Nonhybridized probe DNA is removed by gentle washing. The detection complex is added in order to determine if the probe has found its target. The reaction between the target-bound enzyme complex and a colorless substrate is allowed to proceed in the presence of a suitable chromogen. The formation of a colored compound, indicative of the hybridization of probe to target DNA, is detected with the aid of a light microscope.

Kelker said that more recently, Enzo scientists have developed a method for directly labeling DNA probes with fluorescent molecules. In this procedure, the nucleotide analog, allylamine-dUTP is first incorporated into DNA by nick translation. This derivatized DNA is then reacted with the isothiocyanate form of either fluorescein or rhodamine to yield a fluorescently labeled DNA probe. This fluorescence tachnique for DNA hybridization assays allows the viewing of the results of a hybridization assay immediately after washing the hybridized specimen. This results in the production of a simpler assay. Also, detection by fluorescence is not complicated by the presence of colored compounds in the specimen, such as hemoglobin in blood or other tissue specimens. Kelker said that this technology should also facilitate the development of assays for the simultaneous detection of different pathogens in a single clinical specimen, since it is now possible to label pathogen-specific DNA probes with a variety of distinguishable fluorescent molecules.

Detection of Human Papillomavirus in Biopsy Specimens

The high sensitivity and specificity of nonradioactive *in situ* DNA hybridization assays for virus detection are also illustrated by the ability of this technique to detect HPV DNA in frozen or paraffin-embedded ano-genital biopsy specimens. Kelker said that the development of cervical carcinoma, as well as other ano-genital carcinomas in both males and females, has been correlated with the presence of HPV types 16, 18, 31, and 35 DNA. This has led to the suggestion that patients infected with these HPV types fall into a high-risk category for developing lower genital tract malignancies. Patients infected with HPV types 6 and 11 fall into a lower risk category for developing cancer than patients with HPV 16, 18, 31, or 35, because ano-genital lesions which contain DNA from HPV types 6 and 11 rarely progress from benign to malignant conditions in otherwise healthy individuals, according to Kelker. Thus, the ability to distinguish between different HPV types will help physicians to predict the likelihood that ano-genital lesions will progress to malignant status and to evaulate the effectiveness of newly developed therapeutic treatments.

DNA hybridization studies have been used successfully to identify HPV DNA sequences in benign, premalignant, and malignant lesions in a variety of clinical specimens. However, according to Kelker, many of these DNA hybridization tests are not suitable for large-scale clinical uses because they are lengthy, cumbersome, and use radioactive materials. Even hybridization tests described in the literature which employ nonradioactive detection systems rather than radioactive ones, are not sufficiently simple or rapid to be used in clinical laboratories, according to Kelker.

Kelker said that he and his coworkers have developed a rapid, sensitive, and specific nonradioactive *in situ* hybridization test for detecting HPV 6/11 and HPV 16 DNA in formalin-fixed, paraffin-embedded, and frozen cervical biopsy specimens. The procedure used in these experiments was similar to that outlined previously, except that a short proteolytic digestion step (using proteinase K) and peroxidase treatment of the specimen were added to the protocol. Kelker said that protease digestion was essential to achieve maximum hybridization of HPV probe DNA to the target sequences in formalin-fixed specimens. Peroxidase treatment reduces the level of background staining in these assays by quenching the endogenous peroxidase activities present in the tissue.

This new assay was used to examine 100 biopsy specimens with histological characteristics ranging from condyloma to invasive carcinoma. The sensitivity and specificity of these tests were determined using Southern blot analyses as the reference method. Kelker found that their *in situ* hybridization assay compared favorably with Southern blot assays. Side-by-side comparisons of positive assay results and those obtained using negative controls (such as hybridization experiments performed using nonhomologous HPV DNA probes) were carried out using serial sections from every tissue to facilitate comparison of positive and negative outcomes. Kelker said that the differences between positive and negative assay results were striking. Hybridization of probes to their respective HPV types was absolutely specific. The sensitivity and specificity levels of these in situ hybridization

tests for HPV types 6/11 and 16 were 88 to 89 percent and 99 percent, respectively.

Kelker said that the colorimetric DNA probe for HPV infections is ideally suited for use in clinical laboratories, since reliable results can be obtained in approximately 1 hour after tissue processing and pretreatment. In addition, this in situ assay permits determination of the presence and typing of HPV DNA, as well as the cytological features of cells in a single section of biopsied material. Kelker said that it would be useful for routine and retrospective determinations of HPV DNA in anogenital biopsy specimens. As such, the *in situ* DNA hybridization test described here represents an improvement over other hybridization assay formats, such as Southern blot or dot blot analyses, for the detection of genital HPV infections.

Detection of Other Viral Pathogens

Kelker and his group have also applied this *in situ* hybridization technology to the detection of herpes simplex viruses (HSV types 1 and 2) and cytomegalovirus (CMV).

Genital herpes is a common sexually transmitted disease that results from infection with HSV. Kelker and coworkers have developed a rapid, sensitive, and specific 1-hour culture confirmation test for herpes (ColorGene DNA hybridization test for HSV confirmation). This test consists of the *in situ* hybridization of a biotinylated DNA probe specific for HSV types 1 and 2 to DNA in HSV-infected cells and the detection of the hybridized probe with an avidin-horseradish peroxidase (HRP) complex. The nuclei of HSV-infected cells are stained red using this assay whereas the nuclei of uninfected cells are not stained. Kelker said that clinical studies have shown that the sensitivity and specificity of this test are 100 percent.

Kelker said that he and his group have also developed a direct lesion test for HSV. In this test, *in situ* hybridization using biotinylated HSV probe DNA is performed directly on material taken from suspected herpes lesions. This assay, which eliminates virus culture steps, is currently at the clinical trial stage of development, according to Kelker.

CMV, another member of the herpes virus family, can result in severe clinical manifestations (i.e., pneumonia, mental retardation) or death in neonates and immunocompromised individuals, but usually does not result in severe illnesses in healthy adults. In standard culture tests for CMV the virus is identified by the development of characteristic cytopathic effects in the nuclei of cultured human fibroblasts innoculated with CMV-containing clinical specimens. This method is extremely sensitive. However, 4 to 6 weeks of culture time may be required for conclusive demonstration of the presence of CMV in these specimens. Moreover, these tests are unable to detect the presence of latent CMV infections.

As a prelude to the development of direct specimen tests for CMV, Kelker and coworkers developed a simple and rapid (1-hour) culture confirmation test for CMV, which has a sensitivity and specificity of 100 percent, according to Kelker. The protocol used for this culture test is similar to that described for HSV. Both fluorescent and enzyme-linked color generating systems for detecting the presence of CMV DNA in CMV-infected cells have been successfully applied to this confirmation test. Kelker said that this assay has also been adapted for the detection of CMV DNA in formalin-fixed, paraffin-embedded tissue sections. In a slide shown by Kelker, a red stain in the nuclear inclusions of CMV-infected cells in a liver biopsy specimen was clearly visible following *in situ* hybridization of biotinylated CMV-specific DNA probes and detection using an avidin-HRP color-generating system. Moreover, cellular morphology was faithfully preserved.

Kelker summarized his talk by saying that he has demonstrated the efficacy and desirability of using nonradioactive *in situ* DNA hybridization assays for diagnosis of a variety of viral pathogens. These assays are amenable for use in both culture and direct testing formats. In addition, the simplicity, rapidity, and reliability of these *in situ* DNA hybridization assays make them entirely suitable for use in clinical laboratory settings.

NEW AMPEROMETRIC BIOSENSORS

This topic was discussed by B. Yon Hin (The Biotechnology Center, University of Cambridge, UK). Yon Hin essentially presented a review of this area.

He said that biosensor technology is being widely regarded as an exciting developing area that has the potential to revolutionize analytical procedures in many sectors of activity including health care and veterinary medicine; the food, pharmaceutical, bioprocessing and petrochemical industries; environmental monitoring and control; defence; and agriculture. A plethora of analytes are implicated in these diverse applications and current chemical information is acquired via the operation of sophisticated laboratories, often within centralized facilities, which are intensive in both capital and skilled labor and involve inevitable delays in obtaining the analytical information. However, there are many instances where such arrangements are limiting, according to Yon Hin. For example, in medicine where the diagnosis and later treatment of some diseases may depend critically on accurate real-time information, a need is expressed for decentralization of laboratory analyses into the bedside, office, and home environments. In particular, biosensors will form the basis of cheap, sensitive, selective, rapid in response and easy to use devices for acquiring analytical information, according to Yon Hin. Research into biosensor design and application has been directed towards the development of a new generation of analytical sensors that exploit the unique capacity of biological molecules (typically an enzyme, multienzyme

system, organelle, whole microorganism, tissue slice, or immune, binding or receptor protein) to specifically recognize the target analyte and subsequently respond with a change in one or more physicochemical parameters via an appropriate transducing system in intimate contact with the biologically sensitive material. The biochemical interaction may produce a change in proton concentration, release or uptake of gases (CO_2, O_2, NH_3), specific ions (NH_4^+, anions), heat, electrons, or light (optical density, refractive index) which, when generated in close proximity to a suitable transducer, is converted into a quantifiable electrical signal. The nature of the biochemical change determines the type of transducing system used. This may be potentiometric, amperometric, optical, conductimetric, piezoelectric, or thermometric.

Yon Hin said that considerable interest and research effort have been focused on electrochemical sensors, and a number of analytical devices based on electrochemical-measuring principle have been commercialized successfully. Amperometric biosensors combine the specificity and selectivity of the enzyme for its natural substrate with the analytical powers of electrochemical techniques, according to Yon Hin. Typically these transducers involve redox enzymes such as flavoprotein oxidases or nicotinamide-dependent oxidoreductases, which are instrumental in catalyzing electron transfer to a number of clinically significant metabolites including the most intensively studied substrate, glucose, using the enzyme glucose oxidase. The function of the enzyme is to generate a redox species in a stoichiometric relationship to its substrate. This is then detected by electrochemical oxidation or reduction with the sensing electrode poised at a fixed potential; the measured current thus represents the rate of the enzyme reaction.

Yon Hin said that the earliest forms of amperometric biosensors were enzyme electrodes. The use of an oxygen electrode to monitor glucose oxidation was reported in 1962 and the first electrode using entrapped glucose oxidase (GOD) in conjunction with an oxgen electrode was described in 1967. In 1970, the electrochemical oxidation of the hydrogen peroxide produced by the oxidase enzyme was described as a method for detecting the substrate. Since then many successful commercial devices have been sold based on this principle. However, these electrodes are oxygen dependent and therefore can cause problems under conditions of low oxygen tension, according to Yon Hin. She said that the ideal mode of detection would involve direct electron transfer between the enzyme redox active site and the electrode. Unfortunately, this is often inhibited by thermodynamic and steric constraints and to date only very slow electron-transfer reactions of such enzymes have been observed. Consequently, several strategies have been devised to promote electron transfer between the redox catalyst and the electrode.

One successful approach exploits the use of low-molecular-weight redox species, known as mediators, to shuttle electrons from the prosthetic group of the enzyme to the electrode. Yon Hin said that a good mediator should accept electrons rapidly from the reduced biocatalyst and exhibit reversible electrochemistry at a practical electrode. The mediator should be stable in both oxidized and reduced forms and have a sufficiently low redox potential to obviate interference due to oxidation of extraneous species at the electrode surface. Mediators such as quinone, hexacyanoferrate and some organic dyes have been used to couple electrochemically the glucose oxidase reaction to suitable electrodes. More recently, mediation was achieved with a new generation of artificial electron acceptors based on ferrocene derivatives. The oxidized form, the ferricinium ion, acts as a rapid oxidant to a number of flavoproteins including pyruvate oxidase, xanthine oxidase, diaphorase and glucose oxidase. Genetics International Inc., US, has developed an amperometric glucose-sensing electrode that uses a substituted ferrocene adsorbed on graphite foil with GOD covalently attached to the electrode via a carbodiimide linkage. The ferricinium ion replaces oxygen as the cofactor for GOD. Once reduced, the ferricinium ion can be regenerated at the electrode.

Similarly, direct oxidation of the reduced nicotinamide coenzyme (NADH) at conventional electrodes is not favorable, according to Yon Hin. Again, mediators have been used to catalyze efficiently NADH oxidation. Electrode modification also provides a route to optimizing electron exchange between the enzyme active surface and the electrode. Thus, electrodes chemically modified with surface-immobilized redox functionalities such as orthoquinone and meldola blue, for example, were shown to mediate NADH oxidation. Recently Yon Hin and coworkers reported the successful use of inorganic polymer films of nickel hexacyanoferrate, generated electrochemically at porous nickel electrodes, in amperometric assay of oxidoreductase substrates.

Yon Hin said that conducting organic salts constitute a family of new electrode materials used in enzyme electrodes. These materials such as tetrathiafulvalenium tetracyanoquinodimethanide (TTF-TCNQ) and N-methylphenazium tetracyanoquinodimethanide (NMP-TCNQ) have been used for the oxidation of NADH. These electrode materials have also been used for the oxidation of flavoproteins. The mechanism of electron transfer between the enzyme-active centers and organic salts is still a topic of conjecture among the principle researchers in this area, according to Yon Hin.

A new approach to promote electron transfer from redox centers of enzyme to metal electrodes has recently been proposed. It was shown that the electrical communication between the redox enzyme-active site and conventional gold, platinum, and carbon electrodes can be established by chemical modification of the enzyme. The glucose oxidase was modified with ferrocene carboxylic acid, and it was shown that the modified enzyme

will oxidize directly at the electrode surface. This work has been extended to other ferrocenes and it was found that the stability and overpotential properties of the modified GOD can be improved. Thus, according to Yon Hin, this method opens a new route to bioelectronic sensors.

Recently Yon Hin and coworkers in the main group at the Cambridge Biotechnology Center, under the direction of C.R. Lowe, have suggested the entrapment of redox enzymes in electrically conducting polymers as an alternative means of promoting proximity between the enzyme-active site and the electrode surface. This technique of incorporating enzymes into electrodepositable conducting polymer films also allows the localization of biologically active molecules on defined electrodes of any size or geometry, according to Yon Hin. She said that initial studies have provided evidence that glucose oxidase may be incorporated in polypyrrole or poly-N-methylpyrrole films electrochemically deposited on platinum electrodes. In this approach, the enzyme-catalyzed oxidation of glucose was monitored by electrochemical oxidation of hydrogen peroxide at the electrodes. An extension of this immobilizing procedure was the electrodeposition of both the enzyme and the redox mediator, ferrocene, within the polymer matrix in order to promote electron exchange between the reduced enzyme and the electrode. Pyrrole-ferrocene analogs were synthesized and were electrochemically copolymerized with pyrrole monomers at the electrode surface in the presence of enzyme. Yon Hin said that this technique of enzyme deposition within redox-modified polymers provides an elegant means of producing reagentless enzyme electrodes.

APPLICATION OF ELECTROCHEMICAL METHODS TO IMMUNOASSAYS

The current trend away from radioimmunoassays has stimulated interest in the applications of electrochemical detection systems. Many of these assays are more complex than existing colorimetric tests. Some immunoassays have been designed which are merely adaptations of existing assays; others have been designed with suitable electrochemical labels. The merits of these systems was discussed by M.J. Green (Genetics International, Oxon, UK).

Green said that a growing trend away from radioimmunoassay has been stimulated by a greater awareness of safety. Many alternative labels have been investigated, the most frequently employed of which are enzyme and fluorophores. Some of the most commonly used enzymes are listed in Table 4.

Enzyme-linked immunoassays traditionally involve a sample pretreatment stage and a detection system which is either spectrophotometric or colorimetric. With an increasing trend towards simpler, more sensitive assays, many attempts have been made to couple immunoassays to either amperometric or potentiometric detection, since in the former a linear relationship exists between current and analyte concentration whereas in the latter, potential is linked to concentration by a logarithmic relationship.

Table 4. Enzyme labels used in immunoassays.

Horseradish peroxidase
Alkaline phosphatase
B-D-galactosidase
Glucoamylase
Glucose oxidase
Catalase
Urease
Glucose-6-phosphate dehydrogenase
Malate dehydrogenase

Potentiometric Immunoassays

Proteins are polyelectrolytes and therefore have a net electrical charge, except when placed at their isoelectric point. Therefore, in general, if both antibody and antigen have a net electrical charge and antigen-antibody binding is by van der Waals hydrogen bonding and electrostatic forces, the electrical charge of the resulting complex will be different from that of the antibody alone. The feasibility of monitoring changes in potential on binding antibody to antigen has been demonstrated, according to Green, by coating antibodies to human chorionic gonadotropin (HCG). However, Green said, little further evidence has appeared to support this type of "direct measurement" approach.

Enzyme immunoassays have also been coupled to conventional potentiometric electrodes. An example of this approach is an assay whereby horseradish peroxidase (HRP) is used as an enzyme label which acts upon hydrogen peroxide and pyrogallol to produce carbon dioxide. The carbon dioxide can in turn be detected using a potentiometric carbon dioxide electrode. Green said that this principle has been used to construct an enzyme-labeled digoxin assay.

Other labeling techniques have been employed with conventional ion-selective electrodes, according to Green. A potentiometric ionophore-modulation immunoassay has been described whereby a low-molecular-weight drug is coupled to a potassium ionophore which is included in a polyvinyl chloride membrane mounted on a conventional ion-selective electrode. When the electrode is exposed to a constant concentration of potassium a stable background signal is observed. However, when a suitable antibody is added, reversible binding to the antigen-ionophore conjugate molecules at the membrane solution interface results in a potential change that is proportional to the antibody concentration. According to Green none of these potentiometric systems are as yet available as commercial devices.

Amperometric Immunoassays

Green said that the design of amperometric immunoassays has been approached in a number of ways.

These can generally be divided into three classes: (1) the amperometric immunoassay that utilizes the Clark electrode; (2) the use of an enzyme label, for example, glucose-6-phosphate, dehydrogenase or alkaline phosphatase, with electrochemical detection of products; and (3) assays that employ an electrochemically active label.

Green said that the use of a Clark oxygen electrode in an immunoassay is illustrated by an assay for human chorionic gonadotropin (HCG), in which an antibody to HCG is immobilized on to a membrane which is then placed over the electrode and reacted competitively with HCG and catalase-labeled HCG. The probe is then washed and exposed to hydrogen peroxide. Any catalase-HCG that is bound to the membrane will cause disproportionation of the hydrogen peroxide which can be detected by ar increase in cathodic current due to increased oxygen tension.

Enzyme immunoassays using dehydrogenases as labels have also been adapted for use with electrochemical detection, according to Green. In such systems the NADH produced can be oxidized electrochemically. However, to obtain reliable results sample dilution and pretreatment are required, making the assay more complex than its spectrophotometric counterpart.

A number of groups have demonstrated homogeneous amperometric immunoassays using low-molecular-weight electroactive species as labels. On binding of a labeled species to an antibody the electrochemistry of the label is so perturbed that separation of free from bound label is unnecessary, according to Green. One of the earlier examples is of a homogeneous competitive immunoassay for estriol, with mercuric acetate as a label. Labeling is required as estriol is electrochemically inactive between -200 and -1000 mV. Estriol has also been nitrated in both the 2 and 4 position of ring A to give an electroactive product that shows two reduction waves. Addition of estriol-specific antibody results in a decrease in the peak current, suggesting that there is binding of the antigen to the antibody. On addition of unlabeled estriol to this system, an increase in the reduction current is seen once more; this suggested displacement of the labeled estriol from the antibody by the unlabeled species. However, unfortunately, both the above assays suffer from oxygen interference. Green said that to avoid this problem a more judicious choice of label is required that can either be oxidized at low potentials or reduced at potentials where oxygen interference is negligible. An assay has been described for morphine that uses ferrocene as a label. This assay is based upon the perturbation of the electrochemical activity of ferrocene on binding the antibody, which can be reversed on addition of codeine, a morphine analog. Because the oxidation of the ferricinium species is performed at + 500 mV, interference from oxygen is minimal.

Green said that ferrocene and its derivatives have been shown to act as electron acceptors to a number of flavoproteins: this ability is retained on conjugation of the ferrocene to small drug molecules. Immunoassays have been designed based upon the inhibition of the catalytic activity of the drug-ferrocene complex upon binding of antibody. The extent of the reversal of this effect on addition of free drug provides the measured parameter. However, according to Green, this system has been found unsuitable for commercial development into an electrochemical drug immunoassay for a number of reasons:

- Lack of sensitivity over the clinical range for the system studied
- Nonspecific interactions
- High volume of antiserum required
- Small changes in the measured signal.

Green said that a more successful label for electrochemical immunoassays is alkaline phosphatase. This enzyme is particularly useful for two reasons: (1) alkaline phosphatase has a broad substrate specificity, and many different substrates can be designed that become electroactive on dephosphorylation and (2) the high turnover number of the enzyme enables large quantities of product to be accumulated in a short period of time, providing the basis for a sensitive assay.

Green summarized her presentation by saying that of the several different electrochemical assays that she discussed, some were adaptations of existing spectrophotometric assays. The advantages in sensitivity offered by these assays are somewhat negated by the requirement for extensive sample pretreatment. She said that although direct potentiometric immunoassay showed initial promise, no further progress has been made in designing a workable device. Potentiometric assays involving the use of antigens coupled to ionophores are unlikely to be used routinely due to complex sample handling procedures. Green thinks that it is difficult to imagine such electrochemical immunoassays replacing many of the standard enzyme or fluorescence-polarization immunoassays presently used in hospitals. However, many promising results have been obtained from some of the amperometric approaches and it may not be long before their use is more routine in clinical analysis, according to her.

CONCLUSION

Conceptually new approaches for diagnostic procedures such as enzyme-linked immunoassay, strip and thin-film technologies, fluorescence and luminescene immunoassay, DNA probe technology, and biosensors have already and will in the future provide the availability of rapid, sensitive, simple, and cost-effective assays for diagnostic purposes. Although many of the methods discussed in this report still have to be refined and problems eliminated, the future looks very promising for the commercial application of these procedures. Many of the methods described in this report have potential application not only for clinical diagnosis but are also likely to be applicable to agriculture and veterinary medicine and to

the phamaceutical industry. It is evident that the research and development divisions of industrial organizations are intensely involved in applying the information from basic research to marketable products.

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